IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

NIPPON SHINYAKU CO., LTD., Plaintiff, v. SAREPTA THERAPEUTICS, INC., Defendant.	C.A. No. 21-1015 (GBW) **VOLUME 2 (Part 2) (Exhibits 29-33)**
SAREPTA THERAPEUTICS, INC., Defendant and Counter-Plaintiff, v. NIPPON SHINYAKU CO., LTD. and NS PHARMA, INC. Plaintiff and Counter-Defendants.	

JOINT APPENDIX TO CLAIM CONSTRUCTION BRIEF
FOR THE WILTON/UWA PATENTS

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March 20, 2023

EXHIBIT 29

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office

August 27, 2021

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THIS OFFICE OF:

U.S. PATENT: *9,708,361*
ISSUE DATE: *July 18, 2017*

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T. WALLACE
Certifying Officer

US009708361B2

(12) **United States Patent**
Watanabe et al.

(10) **Patent No.: US 9,708,361 B2**
(45) **Date of Patent: Jul. 18, 2017**

(54) **ANTISENSE NUCLEIC ACIDS**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **14/615,504**

(22) Filed: **Feb. 6, 2015**

(65) **Prior Publication Data**

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Related U.S. Application Data

(63) Continuation of application No. 13/819,520, filed as application No. PCT/JP2011/070318 on Aug. 31, 2011, now Pat. No. 9,079,934.

(30) **Foreign Application Priority Data**

Sep. 1, 2010 (JP) 2010-196032

(51) **Int. Cl.**

C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
A61K 31/70	(2006.01)
C12N 15/11	(2006.01)
C12N 15/113	(2010.01)
C07H 21/00	(2006.01)
C12N 5/00	(2006.01)

(52) **U.S. Cl.**

CPC ***C07H 21/04*** (2013.01); ***C07H 21/00*** (2013.01); ***C12N 15/111*** (2013.01); ***C12N 15/113*** (2013.01); *C12N 2310/11* (2013.01); *C12N 2310/315* (2013.01); *C12N 2310/3145* (2013.01); *C12N 2310/321* (2013.01); *C12N 2310/3525* (2013.01); *C12N 2320/33* (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — Sean McGarry

(74) *Attorney, Agent, or Firm* — Drinker Biddle & Reath LLP

(57) **ABSTRACT**

The present invention provides an oligomer which efficiently enables to cause skipping of the 53rd exon in the human dystrophin gene. Also provided is a pharmaceutical composition which causes skipping of the 53rd exon in the human dystrophin gene with a high efficiency.

7 Claims, 19 Drawing Sheets

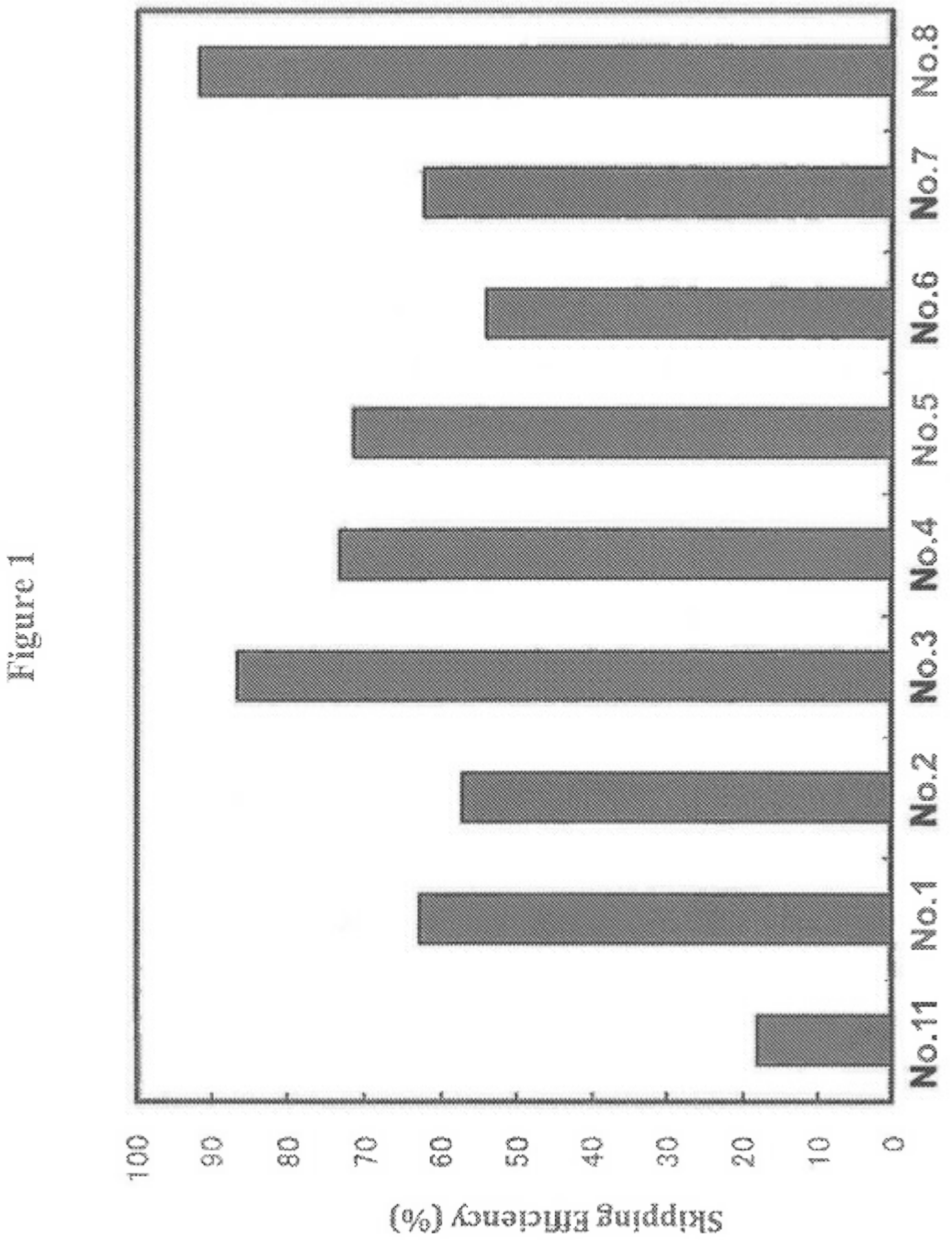
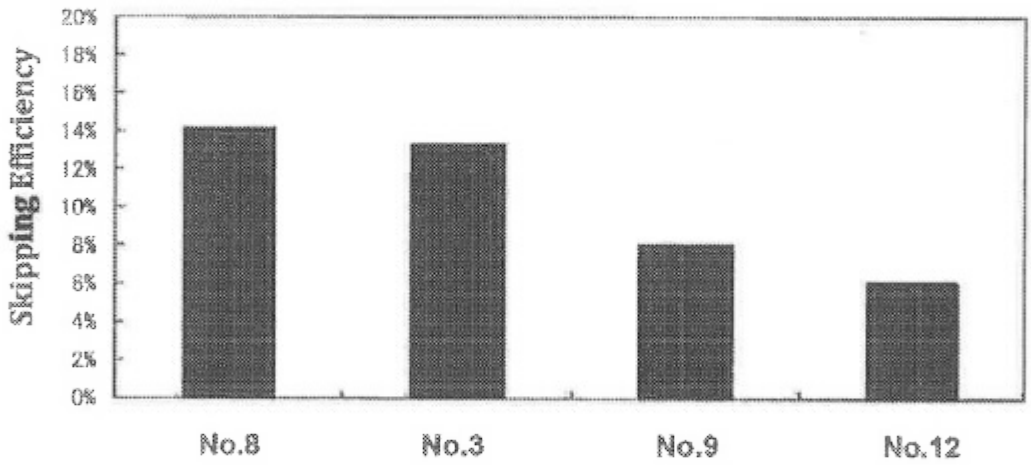


Figure 1

Figure 2



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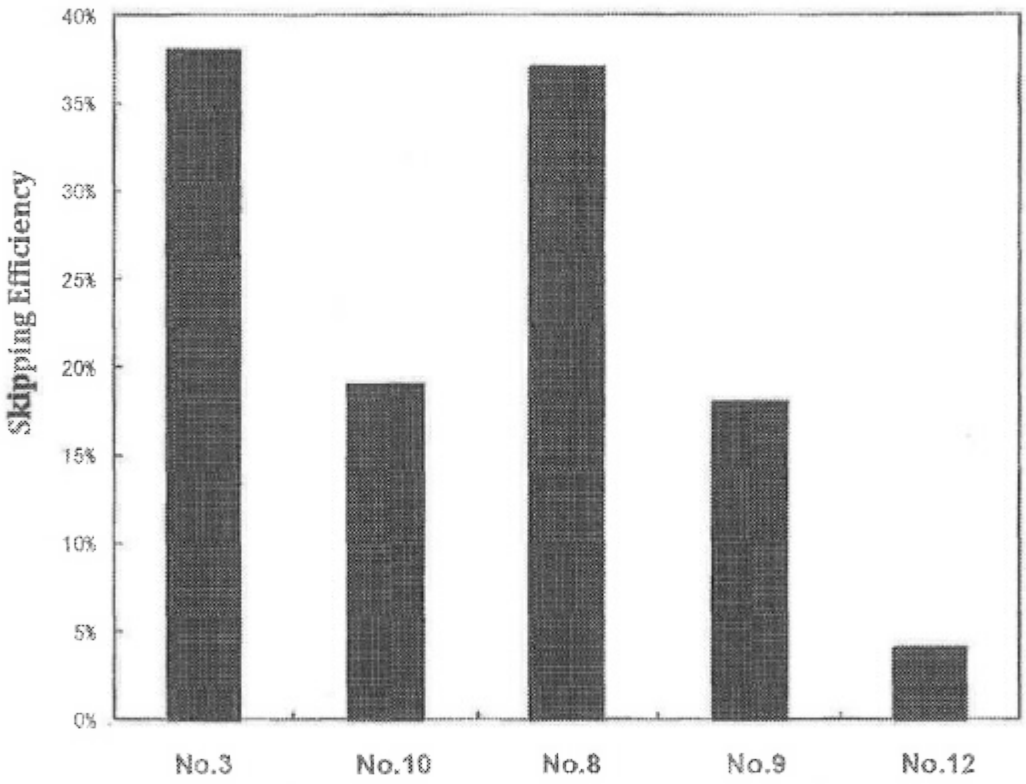

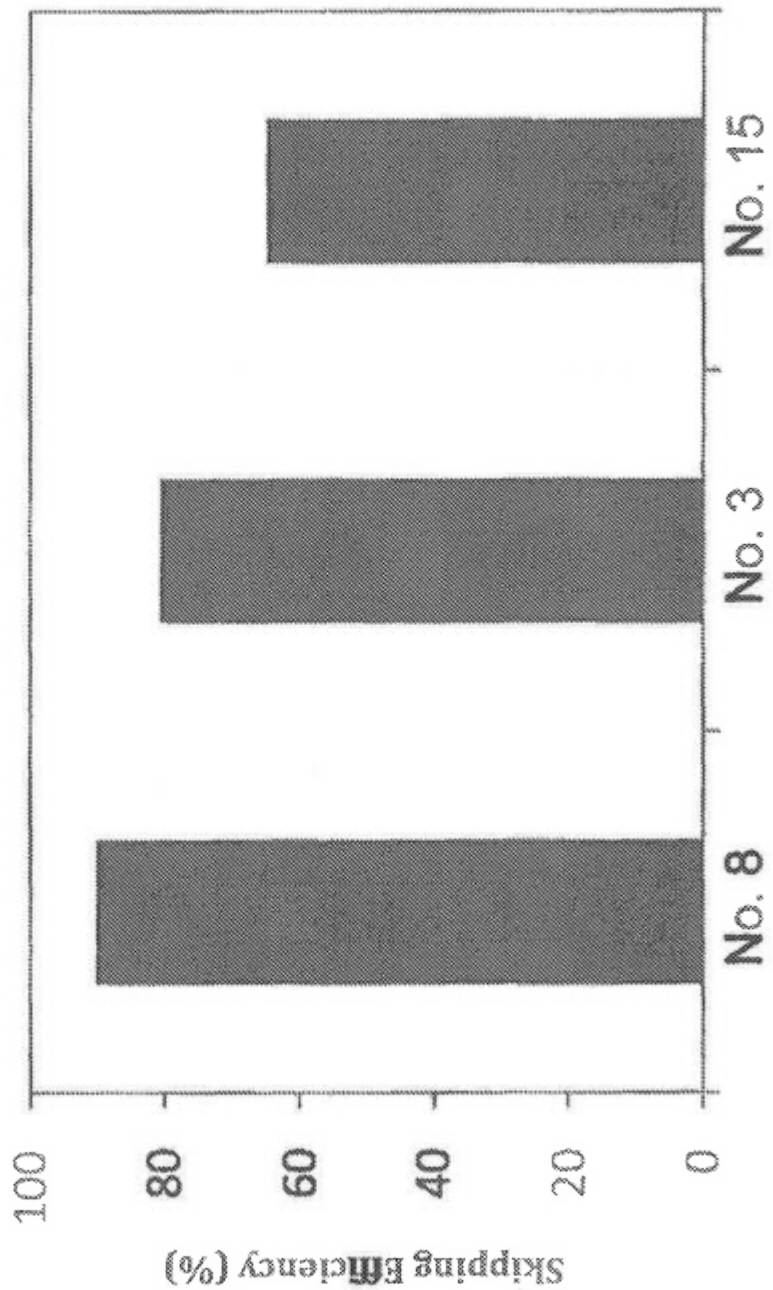

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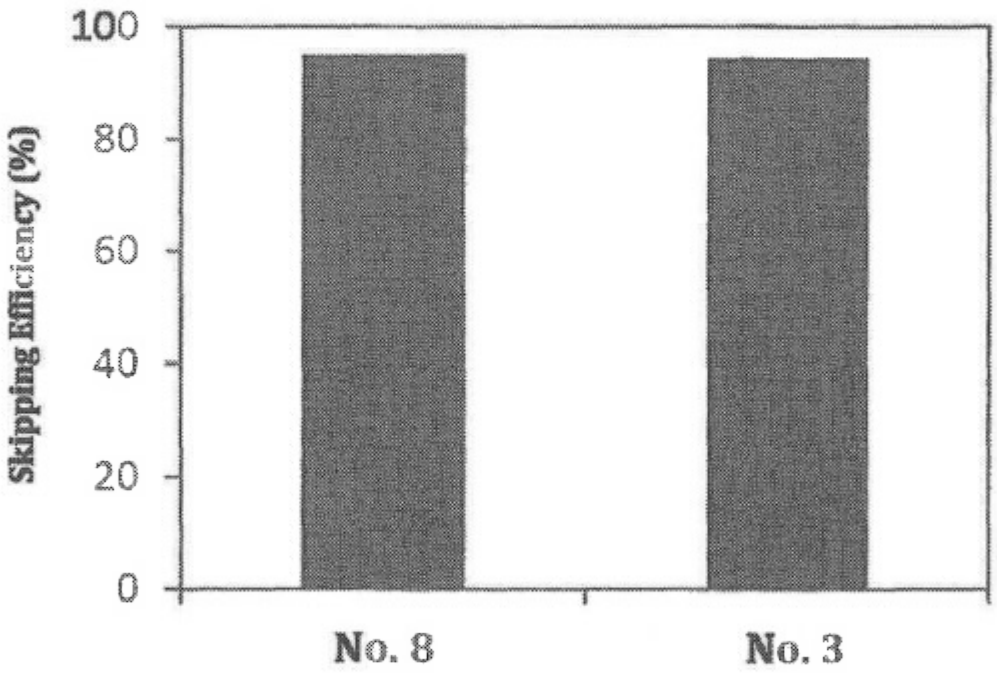

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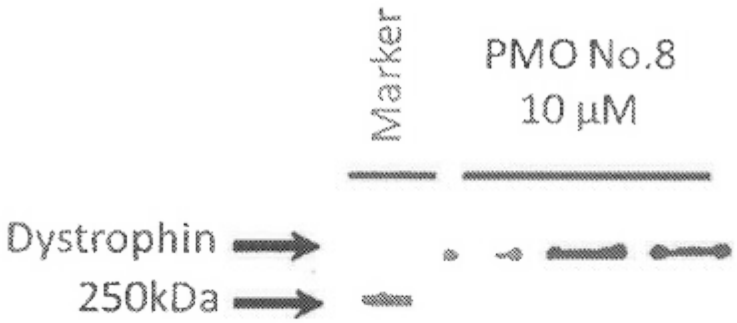

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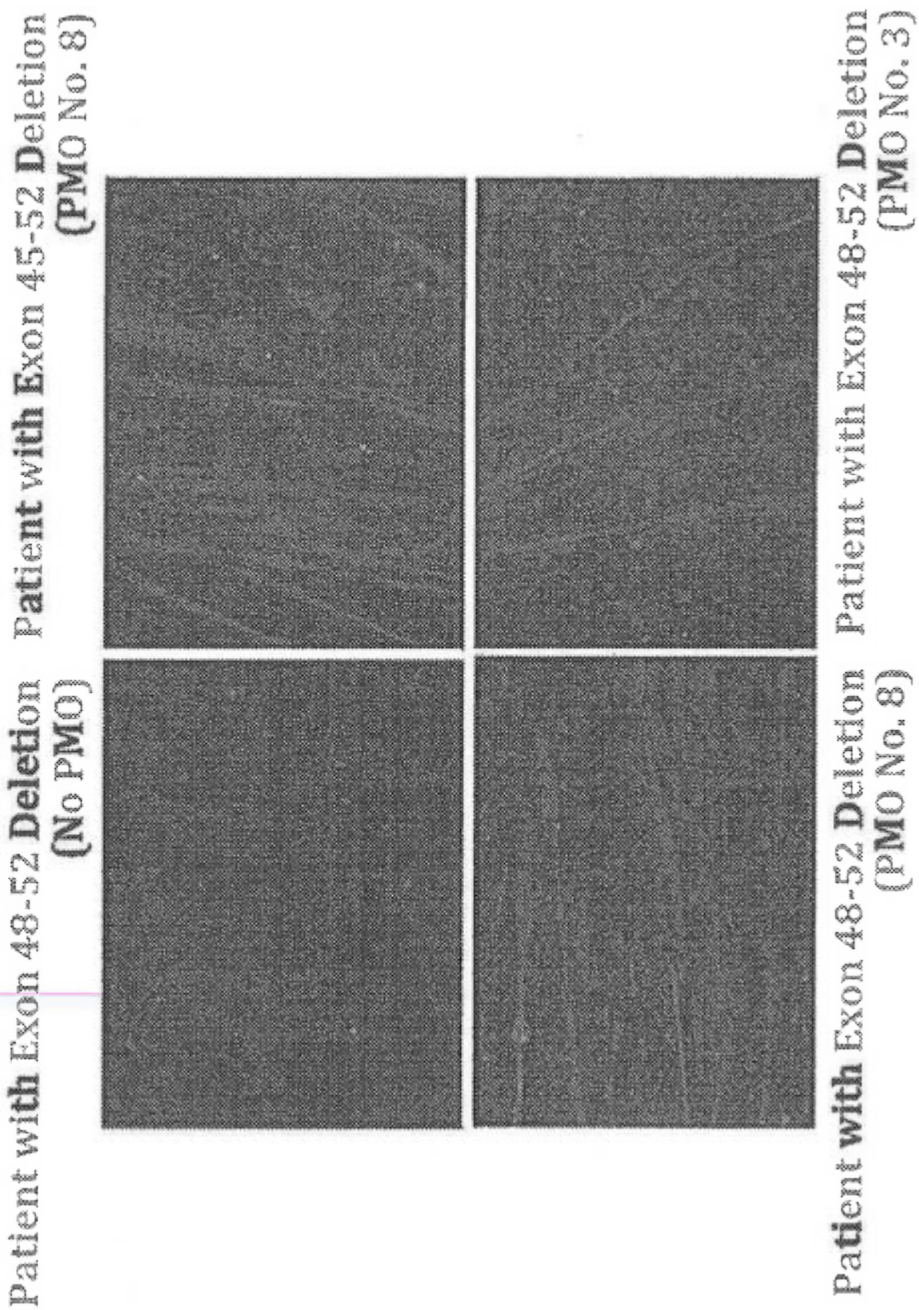

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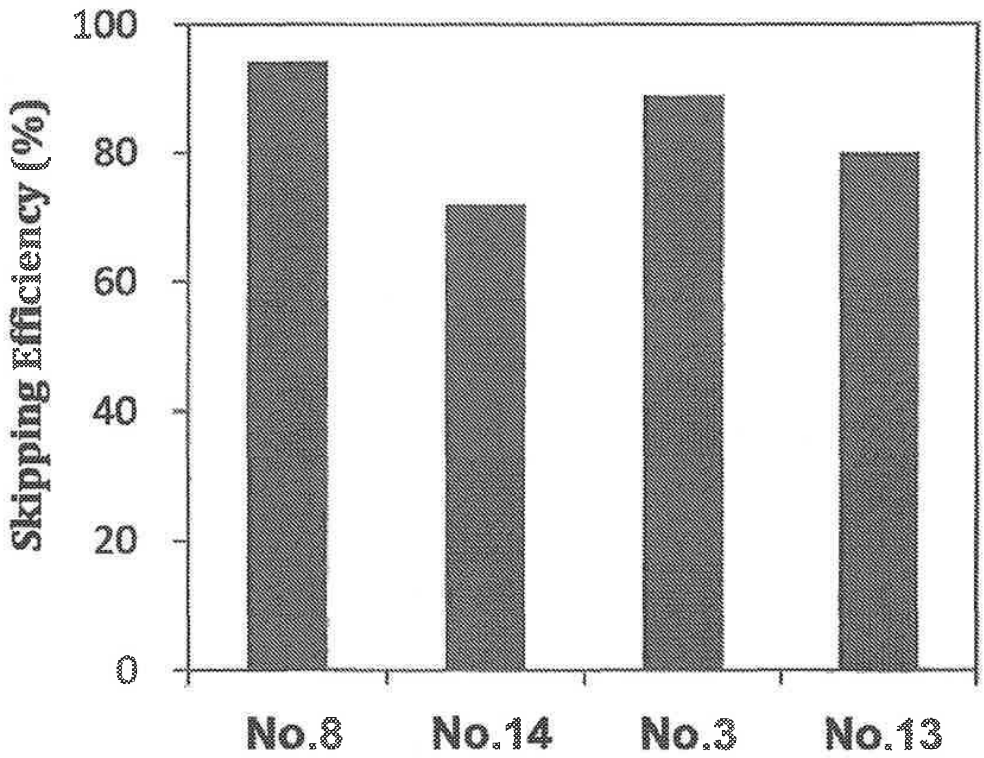

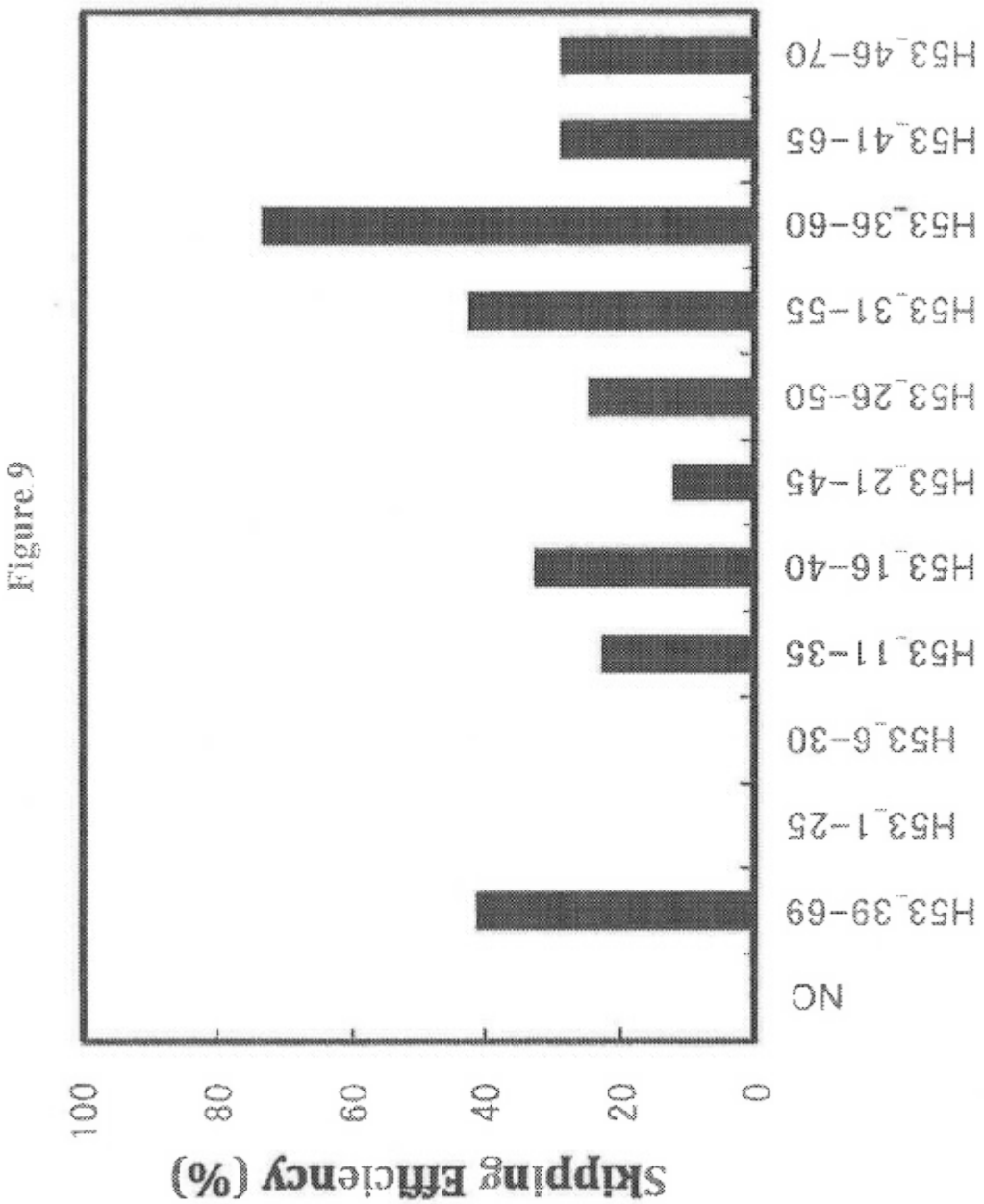

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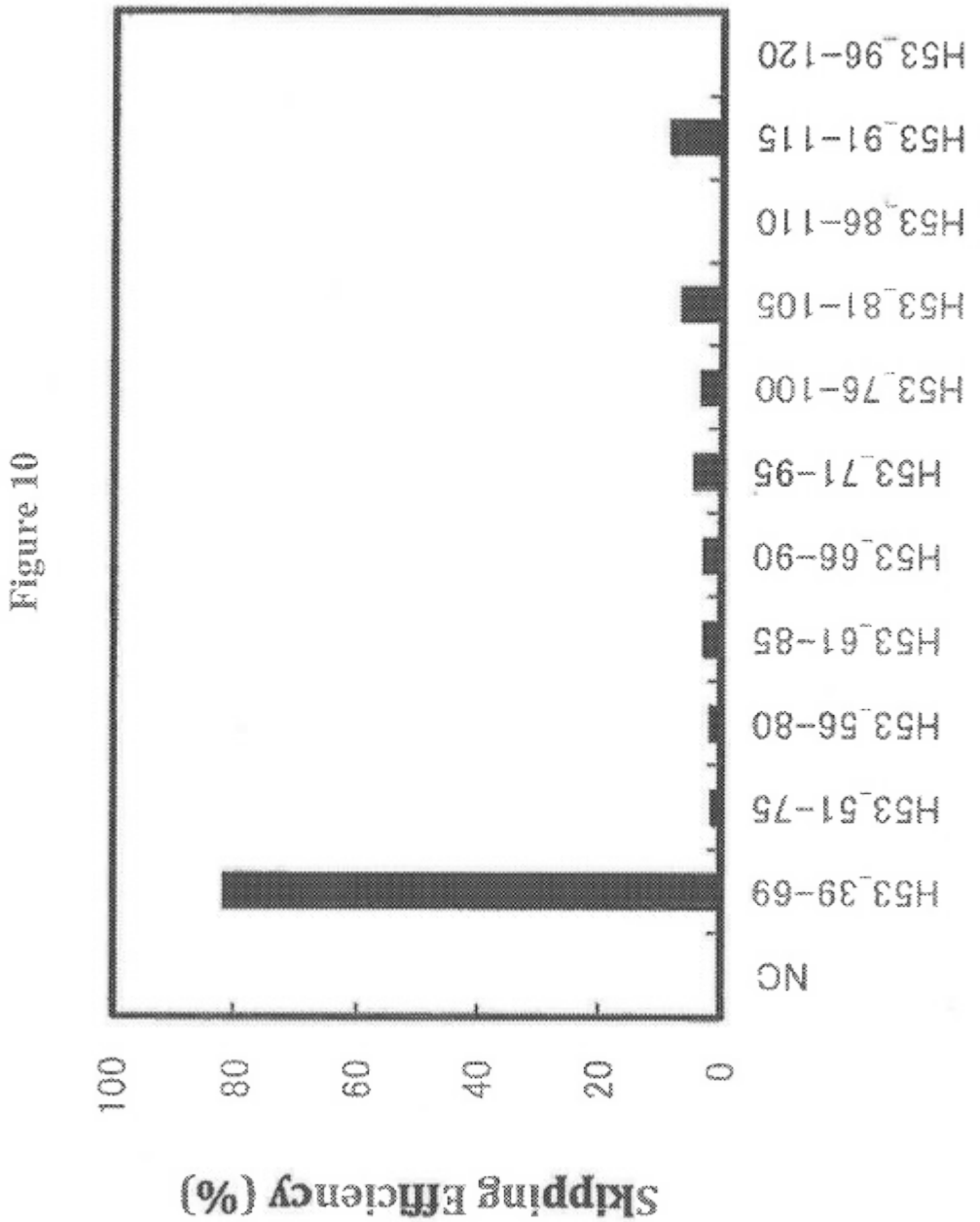

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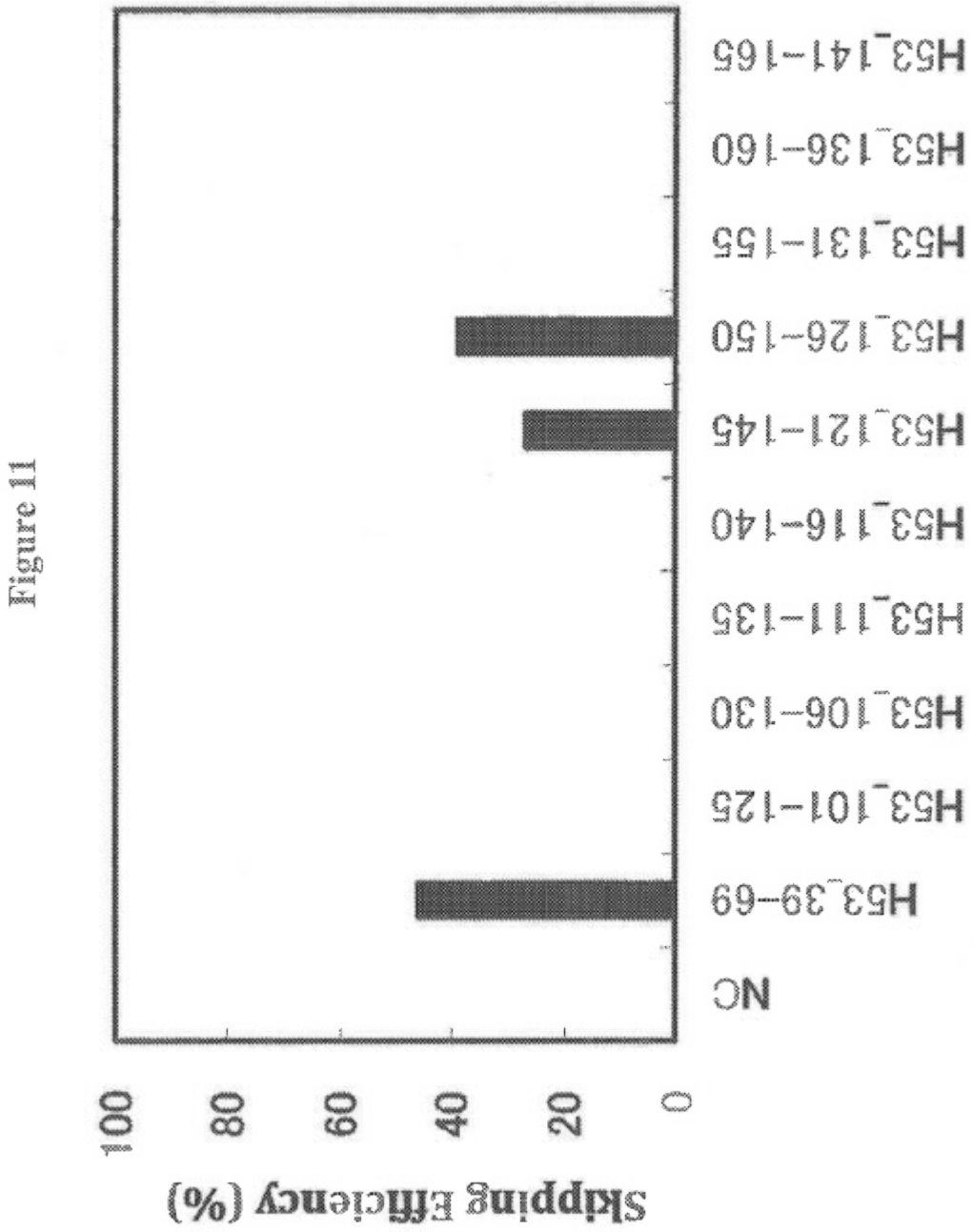

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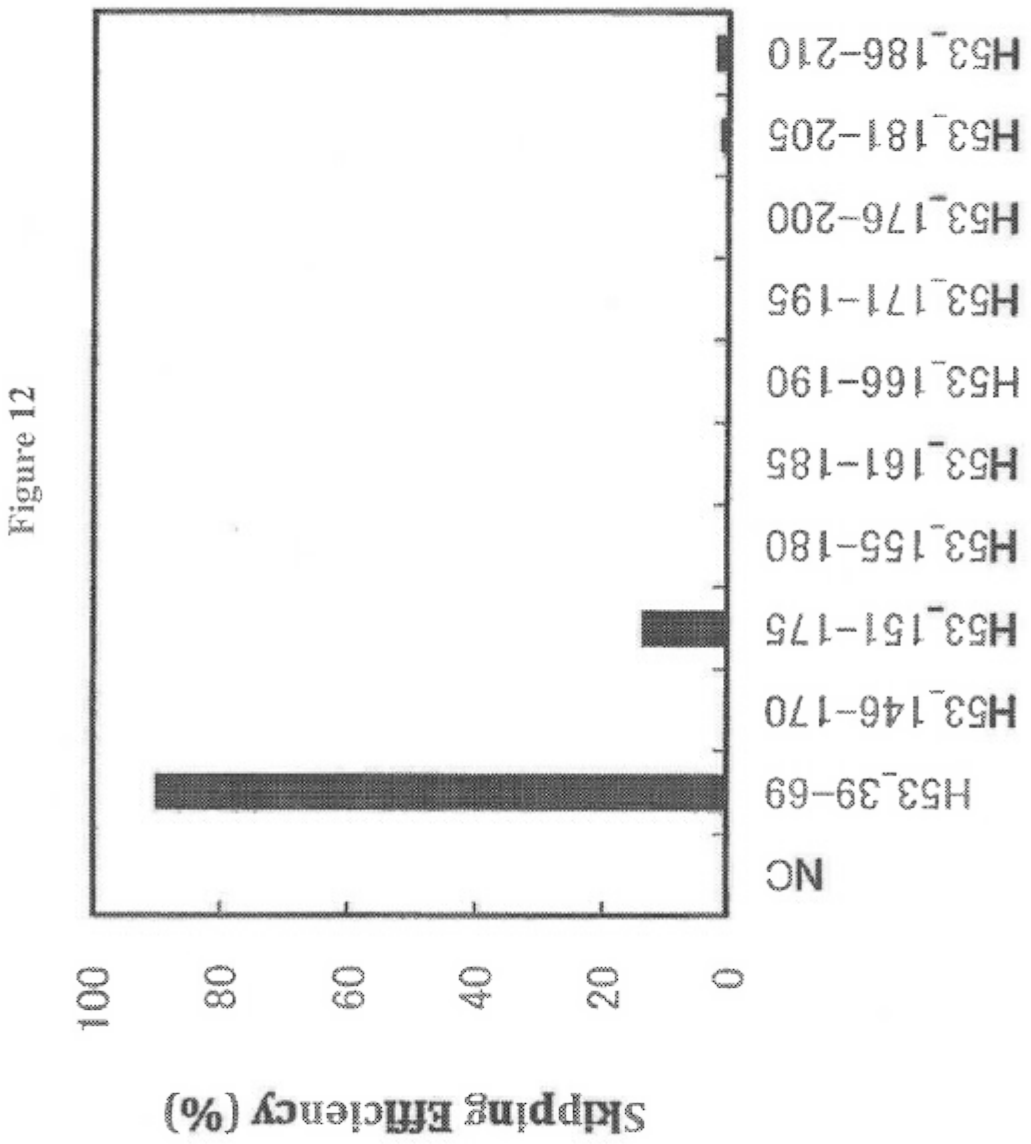

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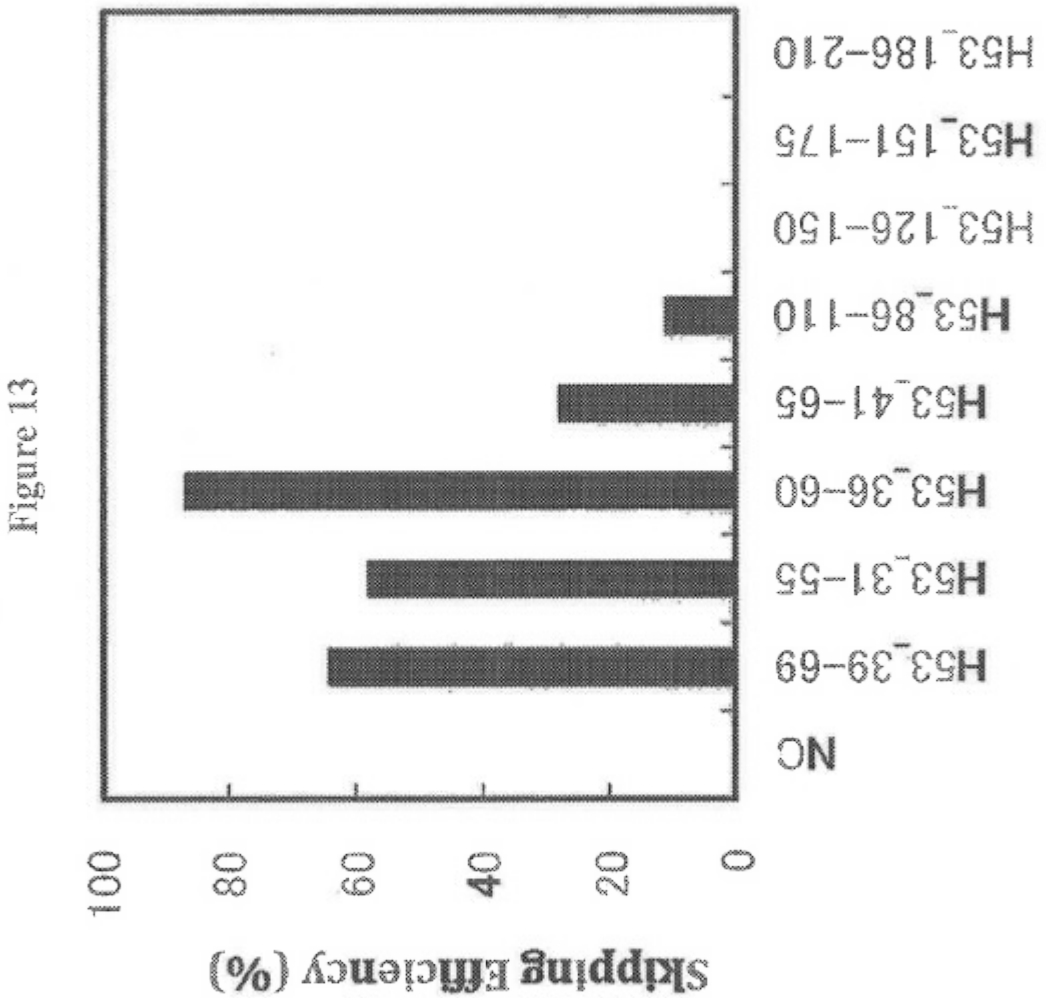

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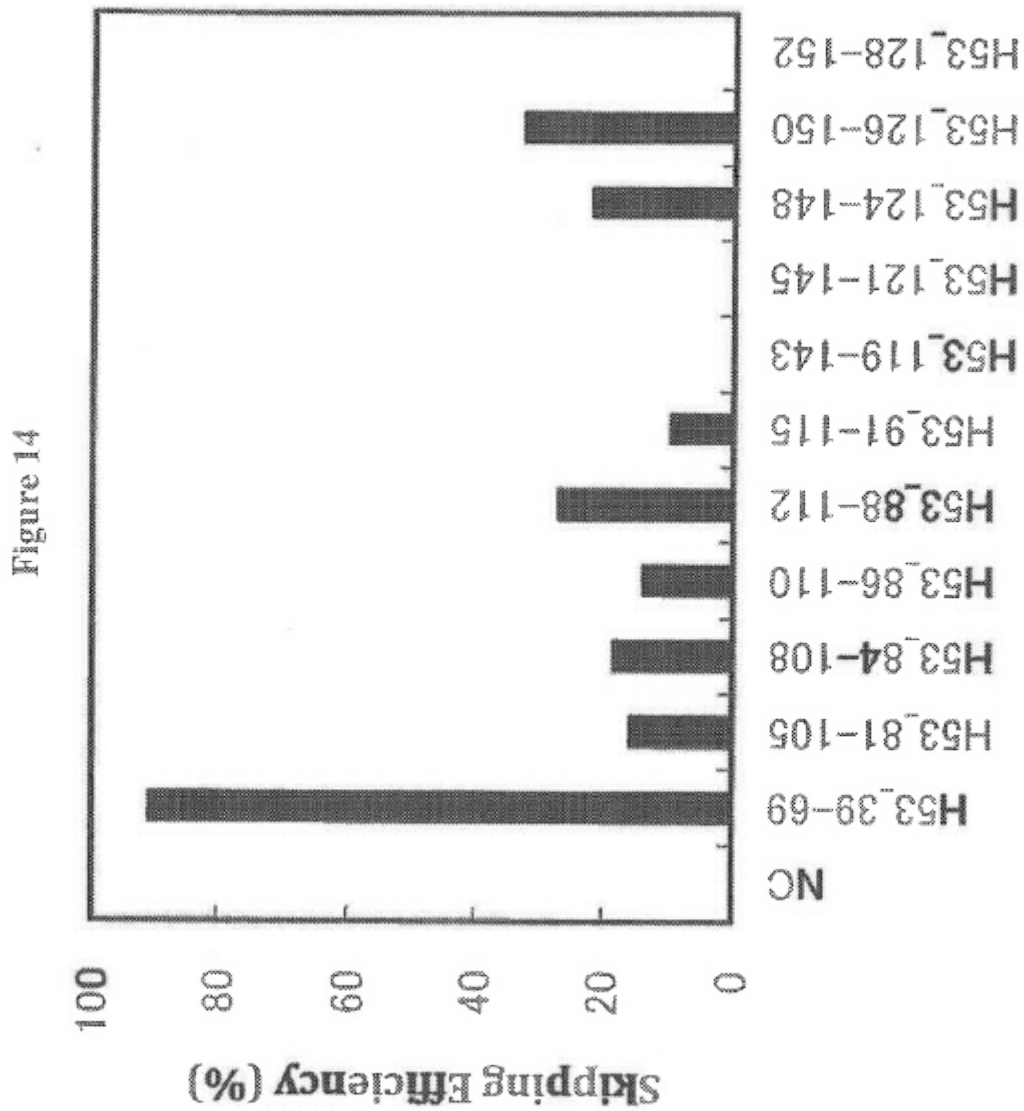

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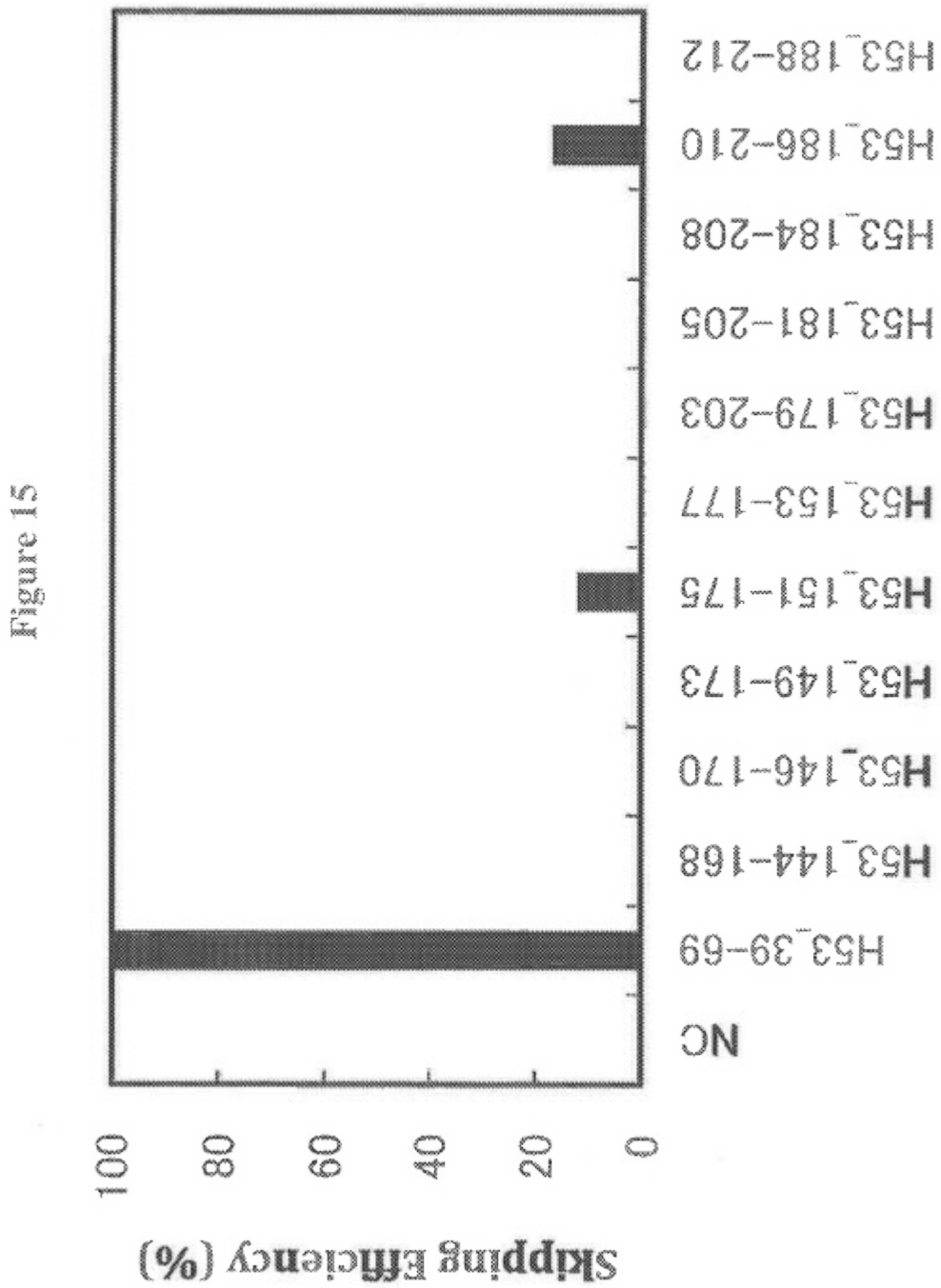

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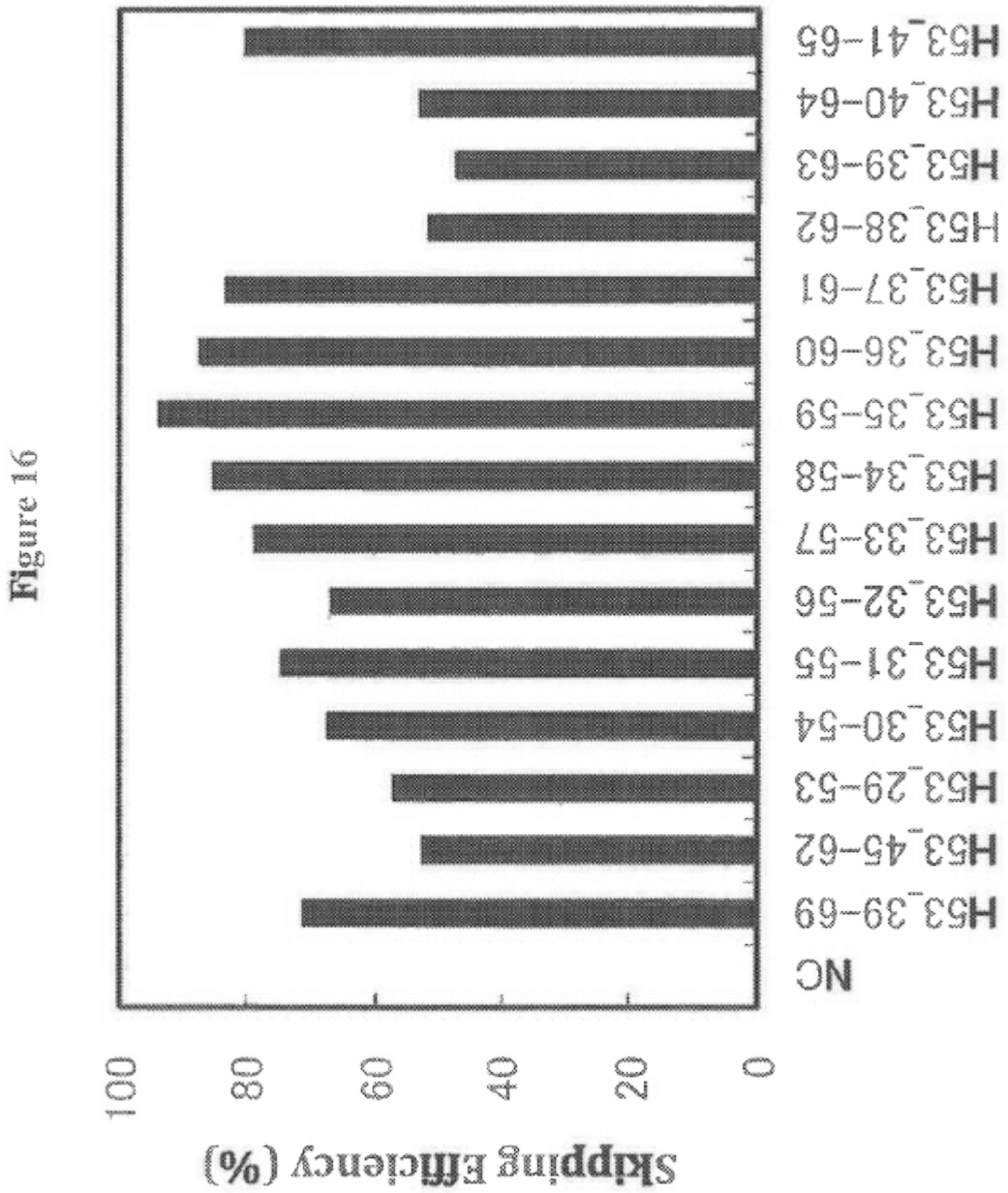

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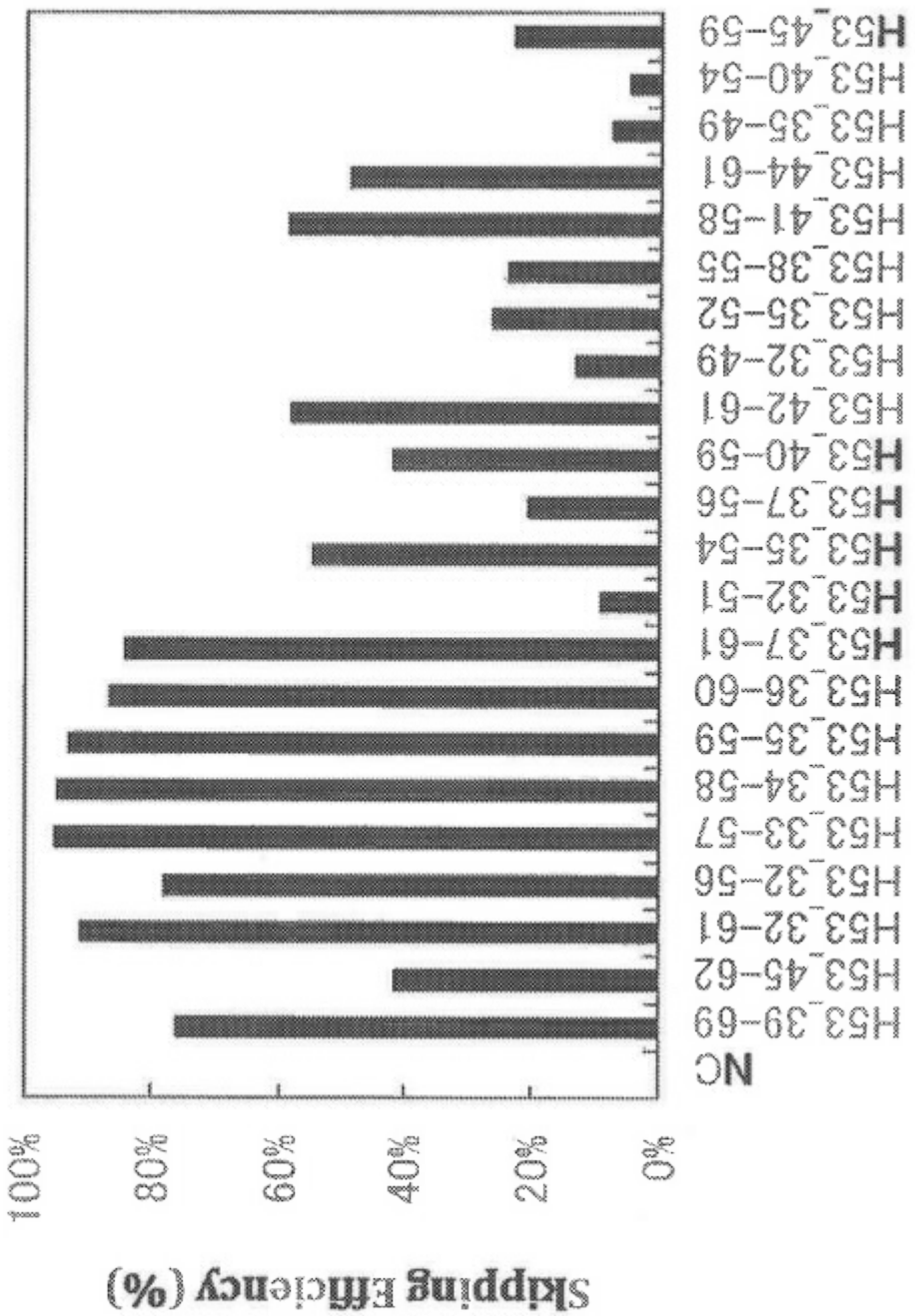

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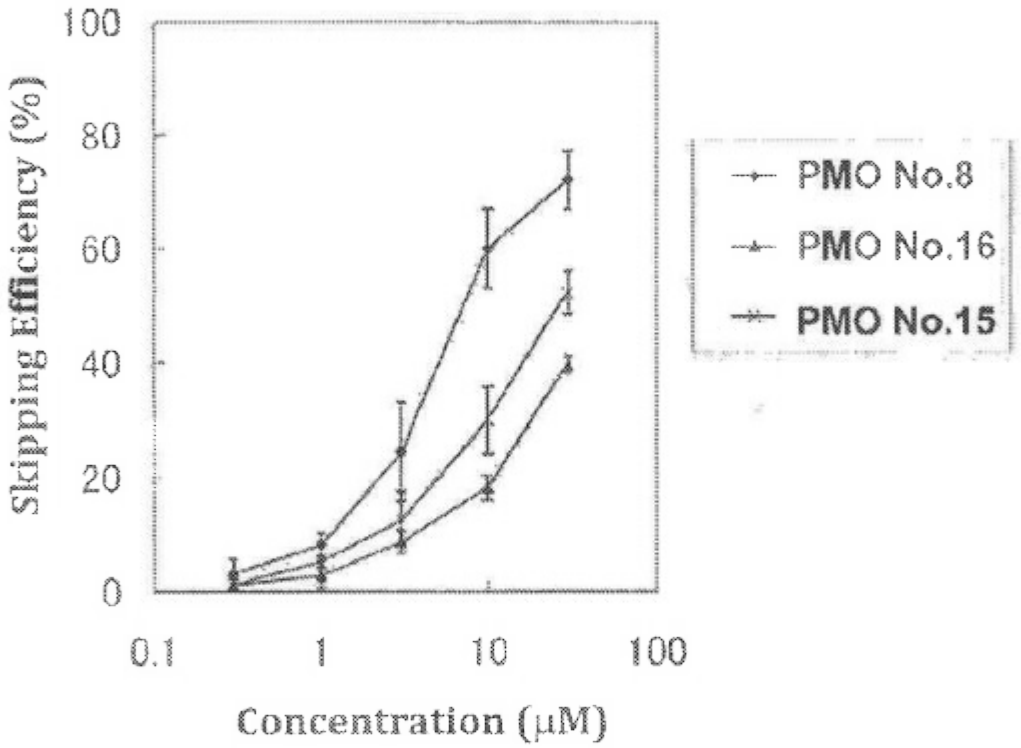


Figure 18

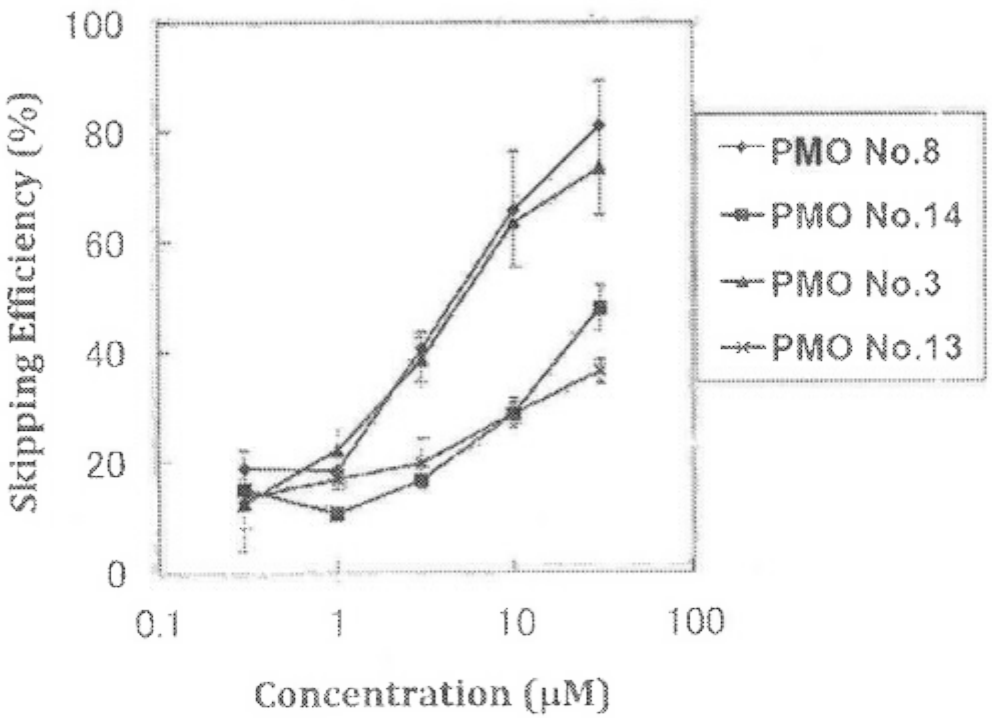


Figure 19

ANTISENSE NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This is a Continuation of copending application Ser. No. 13/819,520, filed Apr. 10, 2013, which is a PCT National Stage of PCT/JP2011/070318 filed Aug. 31, 2011, which claims priority to JP Application No, 2010-196032 filed Sep. 1, 2010.

SEQUENCE LISTING

A Sequence Listing containing SEQ ID NO: 1-123 is incorporated herein by reference.

TECHNICAL FIELD

The present invention relates to an antisense oligomer which causes skipping of exon 53 in the human dystrophin gene, and a pharmaceutical composition comprising the oligomer.

BACKGROUND ART

Duchenne muscular dystrophy (DMD) is the most frequent form of hereditary progressive muscular dystrophy that affects one in about 3,500 newborn boys. Although the motor functions are rarely different from healthy humans in infancy and childhood, muscle weakness is observed in children from around 4 to 5 years old. Then, muscle weakness progresses to the loss of ambulation by about 12 years old and death due to cardiac or respiratory insufficiency in the twenties. DMD is such a severe disorder. At present, there is no effective therapy for DMD available, and it has been strongly desired to develop a novel therapeutic agent.

DMD is known to be caused by a mutation in the dystrophin gene. The dystrophin gene is located on X chromosome and is a huge gene consisting of 2.2 million DNA nucleotide pairs. DNA is transcribed into mRNA precursors, and introns are removed by splicing to synthesize mRNA in which 79 exons are joined together. This mRNA is translated into 3,685 amino acids to produce the dystrophin protein. The dystrophin protein is associated with the maintenance of membrane stability in muscle cells and necessary to make muscle cells less fragile. The dystrophin gene from patients with DMD contains a mutation and hence, the dystrophin protein, which is functional in muscle cells, is rarely expressed. Therefore, the structure of muscle cells cannot be maintained in the body of the patients with DMD, leading to a large influx of calcium ions into muscle cells. Consequently, an inflammation-like response occurs to promote fibrosis so that muscle cells can be regenerated only with difficulty.

Becker muscular dystrophy (BMD) is also caused by a mutation in the dystrophin gene. The symptoms involve muscle weakness accompanied by atrophy of muscle but are typically mild and slow in the progress of muscle weakness, when compared to DMD. In many cases, its onset is in adulthood. Differences in clinical symptoms between DMD and BMD are considered to reside in whether the reading frame for amino acids on the translation of dystrophin mRNA into the dystrophin protein is disrupted by the mutation or not (Non-Patent Document 1). More specifically, in DMD, the presence of mutation shifts the amino acid reading frame so that the expression of functional dystrophin protein is abolished, whereas in BMD the dystrophin protein that functions, though imperfectly, is produced because the amino acid reading frame is preserved, while a part of the exons are deleted by the mutation.

Exon skipping is expected to serve as a method for treating DMD. This method involves modifying splicing to restore the amino acid reading frame of dystrophin mRNA and induce expression of the dystrophin protein having the function partially restored (Non-Patent Document 2). The amino acid sequence part, which is a target for exon skipping, will be lost. For this reason, the dystrophin protein expressed by this treatment becomes shorter than normal one but since the amino acid reading frame is maintained, the function to stabilize muscle cells is partially retained. Consequently, it is expected that exon skipping will lead DMD to the similar symptoms to that of BMD which is milder. The exon skipping approach has passed the animal tests using mice or dogs and now is currently assessed in clinical trials on human DMD patients.

The skipping of an exon can be induced by binding of antisense nucleic acids targeting either 5' or 3' splice site or both sites, or exon-internal sites. An exon will only be included in the mRNA when both splice sites thereof are recognized by the spliceosome complex. Thus, exon skipping can be induced by targeting the splice sites with antisense nucleic acids. Furthermore, the binding of an SR protein to an exonic splicing enhancer (ESE) is considered necessary for an exon to be recognized by the splicing mechanism. Accordingly, exon skipping can also be induced by targeting ESE.

Since a mutation of the dystrophin gene may vary depending on DMD patients, antisense nucleic acids need to be desined based on the site or type of respective genetic mutation. In the past, antisense nucleic acids that induce exon skipping for all 79 exons were produced by Steve Wilton, et al., University of Western Australia (Non-Patent Document 3), and the antisense nucleic acids which induce exon skipping for 39 exons were produced by Annemieke Aartsma-Rus, et al., Netherlands (Non-Patent Document 4).

It is considered that approximately 8% of all DMD patients may be treated by skipping the 53rd exon (hereinafter referred to as "exon 53"). In recent years, a plurality of research organizations reported on the studies where exon 53 in the dystrophin gene was targeted for exon skipping (Patent Documents 1 to 4; Non-Patent Document 5). However, a technique for skipping exon 53 with a high efficiency has not yet been established.

Patent Document 1: International Publication WO 2006/000057

Patent Document 2: International Publication WO 2004/048570

Patent Document 3: US 2010/0168212

Patent Document 4: International Publication WO 2010/048586

Non-Patent Document 1: Monaco A. P. et al., Genomics 1988; 2: p. 90-95

Non-Patent Document 2: Matsuo M., Brain Dev 1996; 18: p. 167-172

Non-Patent Document 3: Wilton S. D., et al., Molecular Therapy 2007: 15: p. 1288-96

Non-Patent Document 4: Annemieke Aartsma-Rus et al., (2002) Neuromuscular Disorders 12: S71-S77

Non-Patent Document 5: Linda J. Popplewell et al., (2010) Neuromuscular Disorders, vol. 20, no. 2, p. 102-10

DISCLOSURE OF THE INVENTION

Under the foregoing circumstances, antisense oligomers that strongly induce exon 53 skipping in the dystrophin gene and muscular dystrophy therapeutics comprising oligomers thereof have been desired.

As a result of detailed studies of the structure of the dystrophin gene, the present inventors have found that exon 53 skipping can be induced with a high efficiency by targeting the sequence consisting of the 32nd to the 56th nucleotides from the 5' end of exon 53 in the mRNA precursor (hereinafter referred to as "pre-mRNA") in the dystrophin gene with antisense oligomers. Based on this finding, the present inventors have accomplished the present invention.

That is, the present invention is as follows.

[1] An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences consisting of the 31st to the 53rd, the 31 st to the 54th, the 31st to the 55th, the 31st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[2] The antisense oligomer according to [1] above, which is an oligonucleotide.

[3] The antisense oligomer according to [2] above, wherein the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified.

[4] The antisense oligomer according to [3] above, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of OR, R, R'OR, SiH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br and I (wherein R is an alkyl or an aryl and R' is an alkylene).

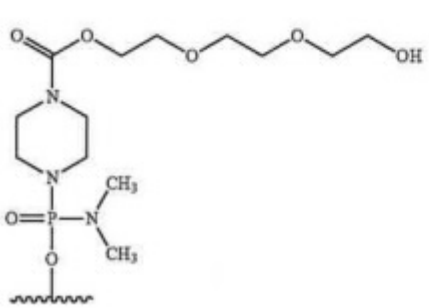
[5] The antisense oligomer according to [3] or [4] above, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond and a boranophosphate bond.

[6] The antisense oligomer according to [1] above, which is a morpholino oligomer.

[7] The antisense oligomer according to [6] above, which is a phosphorodiamidate morpholino oligomer.

[8] The antisense oligomer according to any one of [1] to [7] above, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:

(1)



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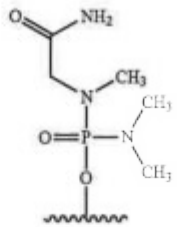

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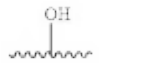


[9] The antisense oligomer according to any one of [1] to [8] above, consisting of a nucleotide sequence complementary to the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides from the 5' end of the 53rd exon in the human dystrophin gene.

[10] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 2 to 37.

[11] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by any one selected from the group consisting of SEQ ID NOS: 11, 17, 23, 29 and 35.

[12] The antisense oligomer according to any one of [1] to [8] above, consisting of the nucleotide sequence shown by SEQ ID NO: 11 or 35.

[13] A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to any one of [1] to [12] above, or a pharmaceutically acceptable salt or hydrate thereof.

The antisense oligomer of the present invention can induce exon 53 skipping in the human dystrophin gene with a high efficiency. In addition, the symptoms of Duchenne muscular dystrophy can be effectively alleviated by administering the pharmaceutical composition of the present invention.

BRIEF DESCRIPTION OF DRAWINGS

FIG. **1** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cell line (RD cells).

FIG. **2** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human normal tissue-derived fibroblasts (TIG-119 cells) to induce differentiation into muscle cells.

FIG. **3** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into human DMD patient-derived fibroblasts (5017 cells) to induce differentiation into muscle cells.

FIG. **4** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **5** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **6** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **7** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52 or deletion of exons 48-52) to induce differentiation into muscle cells.

FIG. **8** shows the efficiency of exon 53 skipping in the human dystrophin gene in the cells where human myoD gene is introduced into fibroblasts from human DMD patient (with deletion of exons 45-52) to induce differentiation into muscle cells.

FIG. **9** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **10** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **11** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **12** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **13** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **14** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **15** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **16** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **17** shows the efficiency of exon 53 skipping (2'-OMe-S-RNA) in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells).

FIG. **18** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

FIG. **19** shows the efficiency of exon 53 skipping in the human dystrophin gene in human rhabdomyosarcoma cells (RD cells) at the respective concentrations of the oligomers.

BEST MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is described in detail. The embodiments described below are intended to be presented by way of example merely to describe the invention but not limited only to the following embodiments. The present invention may be implemented in various ways without departing from the gist of the invention.

All of the publications, published patent applications, patents and other patent documents cited in the specification are herein incorporated by reference in their entirety. The specification hereby incorporates by reference the contents of the specification and drawings in the Japanese Patent Application (No. 2010-196032) filed Sep. 1, 2010, from which the priority was claimed.

1. Antisense Oligomer

The present invention provides the antisense oligomer (hereinafter referred to as the "oligomer of the present invention") which causes skipping of the 53rd exon in the human dystrophin gene, consisting of a nucleotide sequence complementary to any one of the sequences (hereinafter also referred to as "target sequences") consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31 st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th, or the 36th to the 58th nucleotides, from the 5' end of the 53rd exon in the human dystrophin gene.

[Exon 53 in Human Dystrophin Gene]

In the present invention, the term "gene" is intended to mean a genomic gene and also include cDNA, mRNA precursor and mRNA. Preferably, the gene is mRNA precursor, i.e., pre-mRNA.

In the human genome, the human dystrophin gene locates at locus Xp21.2. The human dystrophin gene has a size of 3.0 Mbp and is the largest gene among known human genes. However, the coding regions of the human dystrophin gene are only 14 kb, distributed as 79 exons throughout the human dystrophin gene (Roberts, R G., et al., Genomics, 16: 536-538 (1993)). The pre-mRNA, which is the transcript of the human dystrophin gene, undergoes splicing to generate mature mRNA of 14 kb. The nucleotide sequence of human wild-type dystrophin gene is known (GenBank Accession No. NM_004006).

The nucleotide sequence of exon 53 in the human wild-type dystrophin gene is represented by SEQ ID NO: 1.

The oligomer of the present invention is designed to cause skipping of exon 53 in the human dystrophin gene, thereby modifying the protein encoded by DMD type of dystrophin gene into the BMD type of dystrophin protein. Accordingly, exon 53 in the dystrophin gene that is the target of exon skipping by the oligomer of the present invention includes both wild and mutant types.

Specifically, exon 53 mutants of the human dystrophin gene include the polynucleotides defined in (a) or (b) below.

(a) A polynucleotide that hybridizes under stringent conditions to a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1; and,

(b) A polynucleotide consisting of a nucleotide sequence having at least 90% identity with the nucleotide sequence of SEQ ID NO: 1.

As used herein, the term "polynucleotide" is intended to mean DNA or RNA.

As used herein, the term "polynucleotide that hybridizes under stringent conditions" refers to, for example, a polynucleotide obtained by colony hybridization, plaque hybridization, Southern hybridization or the like, using as a probe all or part of a polynucleotide consisting of a nucleotide sequence complementary to the nucleotide sequence of, e.g., SEQ ID NO: 1. The hybridization method which may be used includes methods described in, for example, "Sambrook & Russell, Molecular Cloning: A Laboratory Manual Vol. 3, Cold Spring Harbor, Laboratory Press 2001,"

"Ausubel, Current Protocols in Molecular Biology, John Wiley & Sons 1987-1997," etc.

As used herein, the term "complementary nucleotide sequence" is not limited only to nucleotide sequences that form Watson-Crick pairs with target nucleotide sequences, but is intended to also include nucleotide sequences which form Wobble base pairs. As used herein, the term Watson-Crick pair refers to a pair of nucleobases in which hydrogen bonds are formed between adenine-thymine, adenine-uracil or guanine-cytosine, and the term Wobble base pair refers to a pair of nucleobases in which hydrogen bonds are formed between guanine-uracil, inosine-uracil, inosine-adenine or inosine-cytosine. As used herein, the term "complementary nucleotide sequence" does not only refers to a nucleotide sequence 100% complementary to the target nucleotide sequence but also refers to a complementary nucleotide sequence that may contain, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target nucleotide sequence.

As used herein, the term "stringent conditions" may be any of low stringent conditions, moderate stringent conditions or high stringent conditions. The term "low stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 32° C. The term "moderate stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 42° C., or 5×SSC, 1% SDS, 50 mM Tris-HCl (pH 7.5), 50% formamide at 42° C. The term "high stringent conditions" are, for example, 5×SSC, 5×Denhardt's solution, 0.5% SDS, 50% formamide at 50° C. or 0.2×SSC, 0.1% SDS at 65° C. Under these conditions, polynucleotides with higher homology are expected to be obtained efficiently at higher temperatures, although multiple factors are involved in hybridization stringency including temperature, probe concentration, probe length, ionic strength, time, salt concentration and others, and those skilled in the art may appropriately select these factors to achieve similar stringency.

When commercially available kits are used for hybridization, for example, an Alkphos Direct Labeling and Detection System (GE Healthcare) may be used. In this case, according to the attached protocol, after cultivation with a labeled probe overnight, the membrane is washed with a primary wash buffer containing 0.1% (w/v) SDS at 55° C., thereby detecting hybridized polynucleotides. Alternatively, in producing a probe based on the entire or part of the nucleotide sequence complementary to the nucleotide sequence of SEQ ID NO: 1, hybridization can be detected with a DIG Nucleic Acid Detection Kit (Roche Diagnostics) when the probe is labeled with digoxigenin (DIG) using a commercially available reagent (e.g., a PCR Labeling Mix (Roche Diagnostics), etc.).

In addition to the polynucleotides described above, other polynucleotides that can be hybridized include polynucleotides having 90% or higher, 91% or higher, 92% or higher, 93% or higher, 94% or higher, 95% or higher, 96% or higher, 97% or higher, 98% or higher, 99% or higher, 99.1% or higher, 99.2% or higher, 99.3% or higher, 99.4% or higher, 99.5% or higher, 99.6% or higher, 99.7% or higher, 99.8% or higher or 99.9% or higher identity with the polynucleotide of SEQ ID NO: 1, as calculated by homology search software BLAST using the default parameters.

The identity between nucleotide sequences may be determined using algorithm BLAST (Basic Local Alignment Search Tool) by Karlin and Altschul (Proc. Natl. Acad. Sci. USA 872264-2268, 1990; Proc. Natl. Acad. Sci. USA 90: 5873, 1993). Programs called BLASTN and BLASTX based on the BLAST algorithm have been developed (Altschul S F, et al: J. Mol. Biol. 215: 403, 1990). When a nucleotide sequence is sequenced using BLASTN, the parameters are, for example, score=100 and wordlength=12. When BLAST and Gapped BLAST programs are used, the default parameters for each program are employed.

Examples of the nucleotide sequences complementary to the sequences consisting of the 31 st to the 53rd, the 31 st to the 54th, the 31 st to the 55th, the 31 st to the 56th, the 31st to the 57th, the 31st to the 58th, the 32nd to the 53rd, the 32nd to the 54th, the 32nd to the 55th, the 32nd to the 56th, the 32nd to the 57th, the 32nd to the 58th, the 33rd to the 53rd, the 33rd to the 54th, the 33rd to the 55th, the 33rd to the 56th, the 33rd to the 57th, the 33rd to the 58th, the 34th to the 53rd, the 34th to the 54th, the 34th to the 55th, the 34th to the 56th, the 34th to the 57th, the 34th to the 58th, the 35th to the 53rd, the 35th to the 54th, the 35th to the 55th, the 35th to the 56th, the 35th to the 57th, the 35th to the 58th, the 36th to the 53rd, the 36th to the 54th, the 36th to the 55th, the 36th to the 56th, the 36th to the 57th and the 36th to the 58th nucleotides, from the 5' end of exon 53.

TABLE 1

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
31-53	5'-CCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 2
31-54	5'-TCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 3
31-55	5'-CTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 4
31-56	5'-CCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 5
31-57	3'-GCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 6
31-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGTA-3'	SEQ ID NO: 7
32-53	5'-CCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 8
32-54	5'-TCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 9
32-55	5'-CTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 10
32-56	5'CCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 11
32-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 12

TABLE 1-continued

Target sequence in exon 53	Complementary nucleotide sequence	SEQ ID NO:
32-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTGT-3'	SEQ ID NO: 13
33-53	5'-CCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 14
33-54	5'-TCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 15
33-55	5'-CTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 16
33-56	5'-CCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 17
33-57	5'-GCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 18
33-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTTG-3'	SEQ ID NO: 19
34-53	5'-CCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 20
34-54	5'TCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 21
34-55	5'-CTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 22
34-56	5'-CCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 23
34-57	5'-GCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 24
34-58	5'-TGCCTCCGGTTCTGAAGGTGTTCTT-3'	SEQ ID NO: 25
35-53	5'-CCGGTTCTGAAGGTGTTCT-'3	SEQ ID NO: 26
35-54	5'-TCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 27
35-55	5'-CTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 28
35-56	5'-CCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 29
35-57	5'-GCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 30
35-58	5'TGCCTCCGGTTCTGAAGGTGTTCT-3'	SEQ ID NO: 31
36-53	5'-CCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 32
36-54	5'-TCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 33
36-55	5'-CTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 34
36-56	5'-CCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 35
36-57	5'-GCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 36
36-58	5'-TGCCTCCGGTTCTGAAGGTGTTC-3'	SEQ ID NO: 37

It is preferred that the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th, the 33rd to the 56th, the 34th to the 56th, the 35th to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11, SEQ ID NO: 17, SEQ ID NO: 23, SEQ ID NO: 29 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

Preferably, the oligomer of the present invention consists of a nucleotide sequence complementary to any one of the sequences consisting of the 32nd to the 56th or the 36th to the 56th nucleotides (e.g., SEQ ID NO: 11 or SEQ ID NO: 35), from the 5' end of the 53rd exon in the human dystrophin gene.

The term "cause skipping of the 53rd exon in the human dystrophin gene" is intended to mean that by binding of the oligomer of the present invention to the site corresponding to exon 53 of the transcript (e.g., pre-mRNA) of the human dystrophin gene, for example, the nucleotide sequence corresponding to the 5' end of exon 54 is spliced at the 3' side of the nucleotide sequence corresponding to the 3' end of exon 51 in DMD patients with deletion of, exon 52 when the transcript undergoes splicing, thus resulting in formation of mature mRNA which is free of codon frame shift.

Accordingly, it is not required for the oligomer of the present invention to have a nucleotide sequence 100% complementary to the target sequence, as far as it causes exon 53 skipping in the human dystrophin gene. The oligomer of the present invention may include, for example, 1 to 3, 1 or 2, or one nucleotide non-complementary to the target sequence.

Herein, the term "binding" described above is intended to mean that when the oligomer of the present invention is mixed with the transcript of human dystrophin gene, both are hybridized under physiological conditions to form a double strand nucleic acid. The term "under physiological conditions" refers to conditions set to mimic the in vivo environment in terms of pH, salt composition and tempera-

ture. The conditions are, for example, 25 to 40° C., preferably 37° C., pH 5 to 8, preferably pH 7.4 and 150 mM of sodium chloride concentration.

Whether the skipping of exon 53 in the human dystrophin gene is caused or not can be confirmed by introducing the oligomer of the present invention into a dystrophin expression cell (e.g., human rhabdomyosarcoma cells), amplifying the region surrounding exon 53 of mRNA of the human dystrophin gene from the total RNA of the dystrophin expression cell by RT-PCR and performing nested PCR or sequence analysis on the PCR amplified product.

The skipping efficiency can be determined as follows. The mRNA for the human dystrophin gene is collected from test cells; in the mRNA, the polynucleotide level "A" of the band where exon 53 is skipped and the polynucleotide level "B" of the band where exon 53 is not skipped are measured. Using these measurement values of "A" and "B," the efficiency is calculated by the following equation:

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

The oligomer of the present invention includes, for example, an oligonucleotide, morpholino oligomer or peptide nucleic acid (PNA), having a length of 18 to 28 nucleotides. The length is preferably from 21 to 25 nucleotides and morpholino oligomers are preferred.

The oligonucleotide described above (hereinafter referred to as "the oligonucleotide of the present invention") is the oligomer of the present invention composed of nucleotides as constituent units. Such nucleotides may be any of ribonucleotides, deoxyribonucleotides and modified nucleotides.

The modified nucleotide refers to one having fully or partly modified nucleobases, sugar moieties and/or phosphate-binding regions, which constitute the ribonucleotide or deoxyribonucleotide.

The nucleobase includes, for example, adenine, guanine, hypoxanthine, cytosine, thymine, uracil, and modified bases thereof. Examples of such modified nucleobases include, but not limited to, pseudouracil, 3-methyluracil, dihydrouracil, 5-alkylcytosines (e.g., 5-methylcytosine), 5-alkyluracils (e.g., 5-ethyluracil), 5-halouracils (5-bromouracil), 6-azapyrimidine, 6-alkylpyrimidines (6-methyluracil), 2-thiouracil, 4-thiouracil, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5'-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, 1-methyladenine, 1-methylhypoxanthine, 2,2-dimethylguanine, 3-methylcytosine, 2-methyladenine, 2-methylguanine, N6-methyladenine, 7-methylguanine, 5-methoxyaminomethyl-2-thiouracil, 5-methylaminomethyluracil, 5-methylcarbonylmethyluracil, 5-methyloxyuracil, 5-methyl-2-thiouracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, 2-thiocytosine, purine, 2,6-diaminopurine, 2-aminopurine, isoguanine, indole, imidazole, xanthine, etc.

Modification of the sugar moiety may include, for example, modifications at the 2'-position of ribose and modifications of the other positions of the sugar. The modification at the 2'-position of ribose includes replacement of the 2'-OH of ribose with OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br or I, wherein R represents an alkyl or an aryl and R' represents an alkylene.

The modification for the other positions of the sugar includes, for example, replacement of O at the 4' position of ribose or deoxyribose with S, bridging between 2' and 4' positions of the sugar, e.g., LNA (locked nucleic acid) or ENA (2'-O,4'-C-ethylene-bridged nucleic acids), but is not limited thereto.

A modification of the phosphate-binding region includes, for example, a modification of replacing phosphodiester bond with phosphorothioate bond, phosphorodithioate bond, alkyl phosphonate bond, phosphoroamidate bond or boranophosphate bond (Enya et al: Bioorganic & Medicinal Chemistry, 2008, 18, 9154-9160) (cf., e.g., Japan Domestic Re-Publications of PCT Application Nos. 2006/129594 and 2006/038608).

The alkyl is preferably a straight or branched alkyl having 1 to 6 carbon atoms. Specific examples include methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, n-pentyl, isopentyl, neopentyl, tert-pentyl, n-hexyl and isohexyl. The alkyl may optionally be substituted. Examples of such substituents are a halogen, an alkoxy, cyano and nitro. The alkyl may be substituted with 1 to 3 substituents.

The cycloalkyl is preferably a cycloalkyl having 5 to 12 carbon atoms. Specific examples include cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl, cyclodecyl and cyclododecyl.

The halogen includes fluorine, chlorine, bromine and iodine.

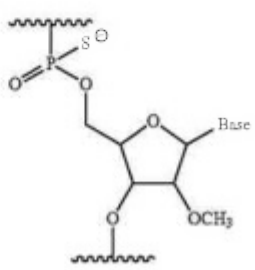
The alkoxy is a straight or branched alkoxy having 1 to 6 carbon atoms such as methoxy, ethoxy, n-propoxy, isopropoxy, n-butoxy, isobutoxy, sec-butoxy, tert-butoxy, n-pentyloxy, isopentyloxy, n-hexyloxy, isohexyloxy, etc. Among others, an alkoxy having 1 to 3 carbon atoms is preferred.

The aryl is preferably an aryl having 6 to 10 carbon atoms. Specific examples include phenyl, α-naphthyl and β-naphthyl. Among others, phenyl is preferred. The aryl may optionally be substituted. Examples of such substituents are an alkyl, a halogen, an alkoxy, cyano and nitro. The aryl may be substituted with one to three of such substituents.

The alkylene is preferably a straight or branched alkylene having 1 to 6 carbon atoms. Specific examples include methylene, ethylene, trimethylene, tetramethylene, pentamethylene, hexamethylene, 2-(ethyl)trimethylene and 1-(methyl)tetramethylene.

The acyl includes a straight or branched alkanoyl or aroyl. Examples of the alkanoyl include formyl, acetyl, 2-methylacetyl, 2,2-dimethylacetyl, propionyl, butyryl, isobutyryl, pentanoyl, 2,2-dimethylpropionyl, hexanoyl, etc. Examples of the aroyl include benzoyl, toluoyl and naphthoyl. The aroyl may optionally be substituted at substitutable positions and may be substituted with an alkyl(s).

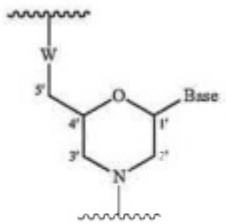
Preferably, the oligonucleotide of the present invention is the oligomer of the present invention containing a constituent unit represented by general formula below wherein the —OH group at position 2' of ribose is substituted with methoxy and the phosphate-binding region is a phosphorothioate bond:



wherein Base represents a nucleobase.

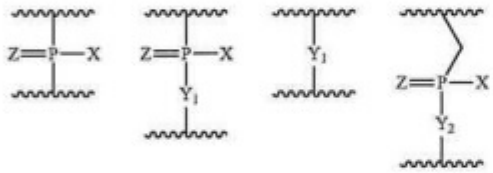
The oligonucleotide of the present invention may be easily synthesized using various automated synthesizer (e.g., AKTA oligopilot plus 10/100 (GE Healthcare)). Alternatively, the synthesis may also be entrusted to a third-party organization (e.g., Promega Inc., or Takara Co.), etc.

The morpholino oligomer of the present invention is the oligomer of the present invention comprising the constituent unit represented by general formula below:



wherein Base has the same significance as defined above, and,

W represents a group shown by any one of the following groups:



wherein

X represents $—CH_2R^1$, $—O—CH_2R^1$, $—S—CH_2R^1$, $—NR_2R^1$ or F;

R^1 represents H or an alkyl;

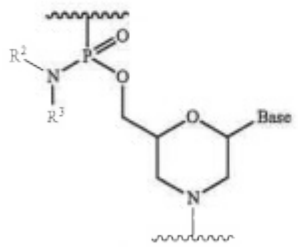
R^2 and R^3, which may be the same or different, each represents H, an alkyl, a cycloalkyl or an aryl;

Y_1 represents O, S, CH_2 or NR^1;

Y_2 represents O, S or NR^1;

Z represents O or S.

Preferably, the morpholino oligomer is an oligomer comprising a constituent unit represented by general formula below (phosphorodiamidate morpholino oligomer (hereinafter referred to as "PMO")).

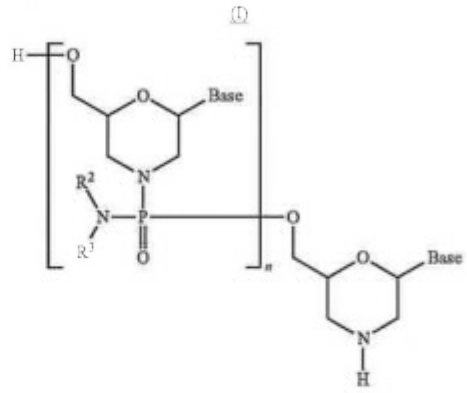


wherein Base, R^2 and R^3 have the same significance as defined above.

The morpholino oligomer may be produced in accordance with, e.g., WO 1991/009033 or WO 2009/064471. In particular, PMO can be produced by the procedure described in WO 2009/064471 or produced by the process shown below.

[Method for Producing PMO]

An embodiment of PMO is, for example, the compound represented by general formula (I) below (hereinafter PMO (I)).



(I)

wherein

Base, R^2 and R^3 have the same significance as defined above; and,

n is a given integer of 1 to 99, preferably a given integer of 18 to 28.

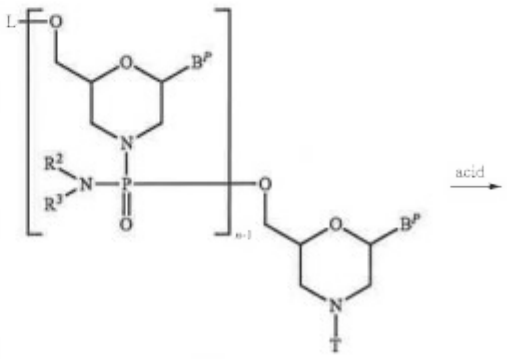
PMO (I) can be produced in accordance with a known method, for example, can be produced by performing the procedures in the following steps.

The compounds and reagents used in the steps below are not particularly limited so long as they are commonly used to prepare PMO.

Also, the following steps can all be carried out by the liquid phase method or the solid phase method (using manuals or commercially available solid phase automated synthesizers). In producing PMO by the solid phase method, it is desired to use automated synthesizers in view of simple operation procedures and accurate synthesis.

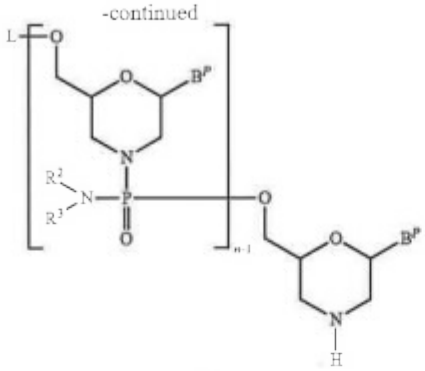
(1) Step A:

The compound represented by general formula (II) below (hereinafter referred to as Compound (II)) is reacted with an acid to prepare the compound represented by general formula (III) below (hereinafter referred to as Compound (III)):



(II)

-continued



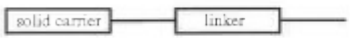
(III)

wherein n, R^2 and R^3 have the same significance as defined above;

each B^P independently represents a nucleobase which may optionally be protected;

T represents trityl, monomethoxytrityl or dimethoxytrityl; and,

L represents hydrogen, an acyl or a group represented by general formula (TV) below (hereinafter referred to as group (IV)).



(IV)

The "nucleobase" for B^P includes the same "nucleobase" as in Base, provided that the amino or hydroxy group in the nucleobase shown by B^P may be protected.

Such protective group for amino is not particularly limited so long as it is used as a protective group for nucleic acids. Specific examples include benzoyl, 4-methoxybenzoyl, acetyl, propionyl, butyryl, isobutyryl, phenylacetyl, phenoxyacetyl, 4-tert-butylphenoxyacetyl, 4-isopropylphenoxyacetyl and (dimethylamino)methylene. Specific examples of the protective group for the hydroxy group include 2-cyanoethyl, 4-nitrophenethyl, phenylsulfonylethyl, methylsulfonylethyl and trimethylsilylethyl, and phenyl, which may be substituted by 1 to 5 electron-withdrawing group at optional substitutable positions, diphenylcarbamoyl, dimethylcarbamoyl, diethylcarbamoyl, methylphenylcarbamoyl, 1-pyrolidinylcarbamoyl, morpholinocarbamoyl, 4-(tert-butylcarboxy)benzyl, 4-[(dimethylamino)carboxy]benzyl and 4-(phenylcarboxy)benzyl, (cf., e.g., WO 2009/064471).

The "solid carrier" is not particularly limited so long as it is a carrier usable for the solid phase reaction of nucleic acids. It is desired for the solid carrier to have the following properties: e.g., (i) it is sparingly soluble in reagents that can be used for the synthesis of morpholino nucleic acid derivatives (e.g., dichloromethane, acetonitrile, tetrazole, N-methylimidazole, pyridine, acetic anhydride, lutidine, trifluoroacetic acid); (ii) it is chemically stable to the reagents usable for the synthesis of morpholino nucleic acid derivatives; (iii) it can be chemically modified; (iv) it can be charged with desired morpholino nucleic acid derivatives; (v) it has a strength sufficient to withstand high pressure through treatments; and (vi) it has a uniform particle diameter range and distribution. Specifically, swellable polystyrene (e.g., aminomethyl polystyrene resin 1% dibenzylbenzene crosslinked (200-400 mesh) (2.4-3.0 mmol/g) (manufactured by Tokyo Chemical Industry), Aminomethylated Polystyrene Resin.HCl [dibenzylbenzene 1%, 100-200 mesh] (manufactured by Peptide Institute, Inc.)), non-swellable polystyrene (e.g., Primer Support (manufactured by GE Healthcare)), PEG chain-attached polystyrene (e.g., NH_2—PEG resin (manufactured by Watanabe Chemical Co.), TentaGel resin), controlled pore glass (controlled pore glass; CPG) (manufactured by, e.g., CPG), oxalyl-controlled pore glass (cf., e.g., Alul et al., Nucleic Acids Research, Vol. 19, 1527 (1991)), TentaGel support-aminopolyethylene glycol-derivatized support (e.g., Wright et al., cf., Tetrahedron Letters, Vol. 34, 3373 (1993)), and a copolymer of Poros-polystyrene/divinylbenzene.

A "linker" which can be used is a known linker generally used to connect nucleic acids or morpholino nucleic acid derivatives. Examples include 3-aminopropyl, succinyl, 2,2'-diethanolsulfonyl and a long chain alkyl amino (LCAA).

This step can be performed by reacting Compound (11) with an acid.

The "acid" which can be used in this step includes, for example, trifluoroacetic acid, dichloroacetic acid and trichloroacetic acid. The acid used is appropriately in a range of, for example, 0.1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (II), preferably in a range of 1 mol equivalent to 100 mol equivalents based on 1 mol of Compound (II).

An organic amine can be used in combination with the acid described above. The organic amine is not particularly limited and includes, for example, triethylamine. The amount of the organic amine used is appropriately in a range of, e.g., 0.01 mol equivalent to 10 mol equivalents, and preferably in a range of 0.1 mol equivalent to 2 mol equivalents, based on 1 mol of the acid.

When a salt or mixture of the acid and the organic amine is used in this step, the salt or mixture includes, for example, a salt or mixture of trifluoroacetic acid and triethylamine, and more specifically, a mixture of 1 equivalent of triethylamine and 2 equivalents of trifluoroacetic acid.

The acid which can be used in this step may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

The reaction temperature in the reaction described above is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

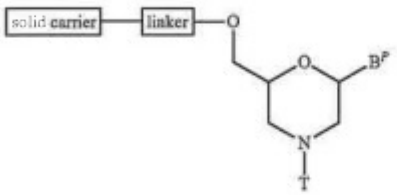
The reaction time may vary depending upon kind of the acid used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

After completion of this step, a base may be added, if necessary, to neutralize the acid remained in the system. The "base" is not particularly limited and includes, for example, diisopropylamine. The base may also be used in the form of a dilution with an appropriate solvent in a concentration of 0.1% (v/v) to 30% (v/v).

The solvent used in this step is not particularly limited so long as it is inert to the reaction, and includes dichloromethane, acetonitrile, an alcohol (ethanol, isopropanol, trifluoroethanol, etc.), water, and a mixture thereof. The reaction temperature is preferably in a range of, e.g., 10° C. to 50° C., more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

In Compound (II), the compound of general formula (IIa) below (hereinafter Compound (IIa)), wherein n is 1 and L is a group (IV), can be produced by the following procedure.

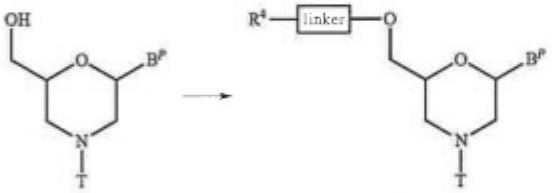


(IIa)

wherein B^P, T, linker and solid carrier have the same significance as defined above.

Step 1

The compound represented by general formula (V) below is reacted with an acylating agent to prepare the compound represented by general formula (VI) below (hereinafter referred to as Compound (VI)).



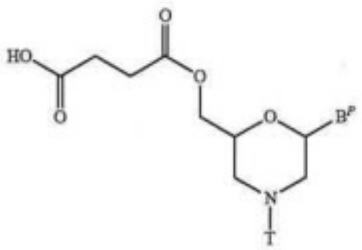
(V) (VI)

wherein B^P, T and linker have the same significance as defined above; and,

R^4 represents hydroxy, a halogen or amino.

This step can be carried out by known procedures for introducing linkers, using Compound (V) as the starting material.

In particular, the compound represented by general formula (VIa) below can be produced by performing the method known as esterification, using Compound (V) and succinic anhydride.

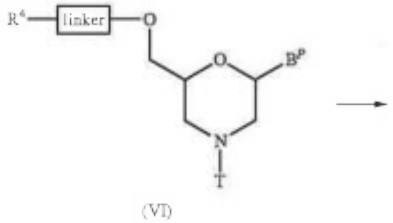


(VIa)

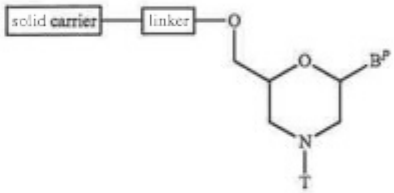
wherein B^P and T have the same significance as defined above.

Step 2

Compound (VI) is reacted with a solid career by a condensing agent to prepare Compound (IIa).



(VI)



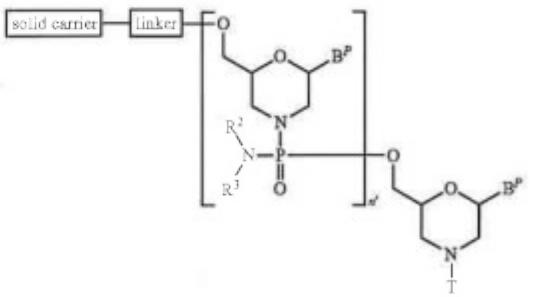
(IIa)

wherein B^P, R^4, T, linker and solid carrier have the same significance as defined above.

This step can be performed using Compound (VI) and a solid carrier in accordance with a process known as condensation reaction.

In Compound (II), the compound represented by general formula (IIa2) below wherein n is 2 to 99 and L is a group represented by general formula (IV) can be produced by using Compound (IIa) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

(IIa2)

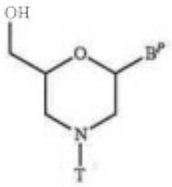


wherein B^P, R^2, R^3, T, linker and solid carrier have the same significance as defined above; and,

n' represents 1 to 98.

In Compound (II), the compound of general formula (IIb) below wherein n is 1 and L is hydrogen can be produced by the procedure described in, e.g., WO 1991/009033.

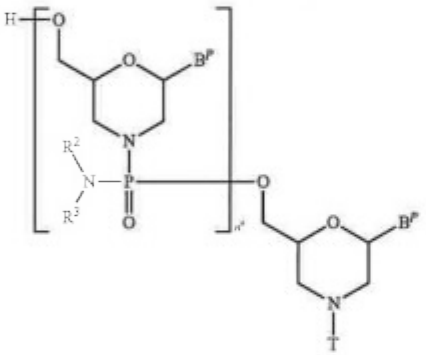
(IIb)



wherein B^P and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIb2) below wherein n is 2 to 99 and L is hydrogen can be produced by using Compound (IIb) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

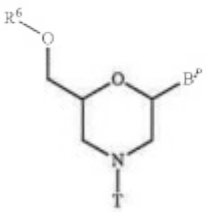
(IIb2)



wherein B^P, n', R^2, R^3 and T have the same significance as defined above.

In Compound (II), the compound represented by general formula (IIc) below wherein n is 1 and L is an acyl can be produced by performing the procedure known as acylation reaction, using Compound (IIb).

(IIc)

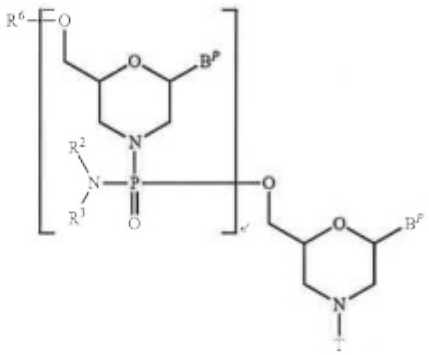


wherein B^P and T have the same significance as defined above; and,

R^5 represents an acyl.

In Compound (II), the compound represented by general formula (IIc2) below wherein n is 2 to 99 and L is an acyl can be produced by using Compound (IIc) as the starting material and repeating step A and step B of the PMO production method described in the specification for a desired number of times.

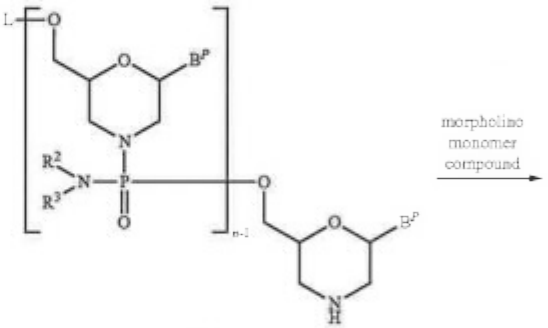
(IIc2)



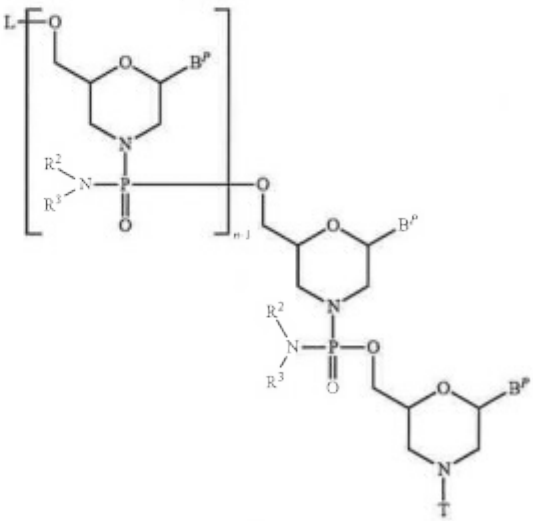
wherein B^P, n', R^2, R^3, R^5 and T have the same significance as defined above.

(2) Step B

Compound (III) is reacted with a morpholino monomer compound in the presence of a base to prepare the compound represented by general formula (VII) below (hereinafter referred to as Compound (VII)):



(III)



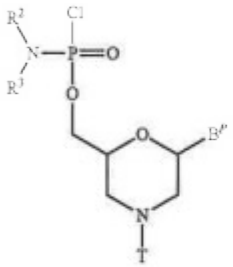
(VII)

wherein B^P, L, n, R^2, R^3 and T have the same significance as defined above.

This step can be performed by reacting Compound (III) with the morpholino monomer compound in the presence of a base.

The morpholino monomer compound includes, for example, compounds represented by general formula (VIII) below:

(VIII)



wherein B^P, R^2, R^3 and T have the same significance as defined above.

The "base" which can be used in this step includes, for example, diisopropylamine, triethylamine and N-ethylmorpholine. The amount of the base used is appropriately in a range of 1 mol equivalent to 1000 mol equivalents based on 1 mol of Compound (III), preferably, 10 mol equivalents to 100 mol equivalents based on 1 mol of Compound (III).

The morpholino monomer compound and base which can be used in this step may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, N,N-dimethylimidazolidone, N-methylpiperidone, DMF, dichloromethane, acetonitrile, tetrahydrofuran, or a mixture thereof.

The reaction temperature is preferably in a range of, e.g., 0° C. to 100° C., and more preferably, in a range of 10° C. to 50° C.

The reaction time may vary depending upon kind of the base used and reaction temperature, and is appropriately in a range of 1 minute to 48 hours in general, and preferably in a range of 30 minutes to 24 hours.

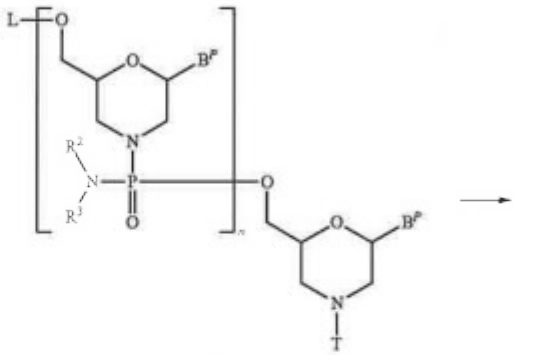
Furthermore, after completion of this step, an acylating agent can be added, if necessary. The "acylating agent" includes, for example, acetic anhydride, acetyl chloride and phenoxyacetic anhydride. The acylating agent may also be used as a dilution with an appropriate solvent in a concentration of 0.1% to 30%. The solvent is not particularly limited as far as it is inert to the reaction, and includes, for example, dichloromethane, acetonitrile, an alcohol(s) (ethanol, isopropanol, trifluoroethanol, etc.), water, or a mixture thereof.

If necessary, a base such as pyridine, lutidine, collidine, triethylamine, diisopropylethylamine, N-ethylmorpholine, etc. may also be used in combination with the acylating agent. The amount of the acylating agent is appropriately in a range of 0.1 mol equivalent to 10000 mol equivalents, and preferably in a range of 1 mol equivalent to 1000 mol equivalents. The amount of the base is appropriately in a range of, e.g., 0.1 mol equivalent to 100 mol equivalents, and preferably in a range of 1 mol equivalent to 10 mol equivalents, based on 1 mol of the acylating agent.

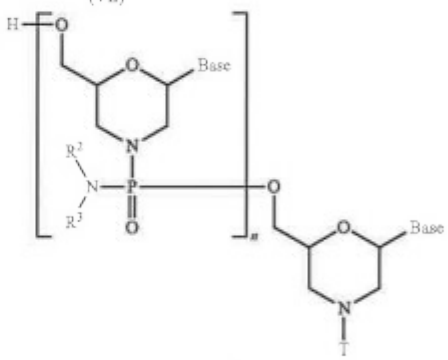
The reaction temperature in this reaction is preferably in a range of 10° C. to 50° C., more preferably, in a range of 10° C. to 50° C., much more preferably, in a range of 20° C. to 40° C., and most preferably, in a range of 25° C. to 35° C. The reaction time may vary depending upon kind of the acylating agent used and reaction temperature, and is appropriately in a range of 0.1 minute to 24 hours in general, and preferably in a range of 1 minute to 5 hours.

(3) Step C:

In Compound (VII) produced in Step B, the protective group is removed using a deprotecting agent to prepare the compound represented by general formula (IX).



(VII)



(IX)

wherein Base, B^P, n, R^2, R^3 and T have the same significance as defined above.

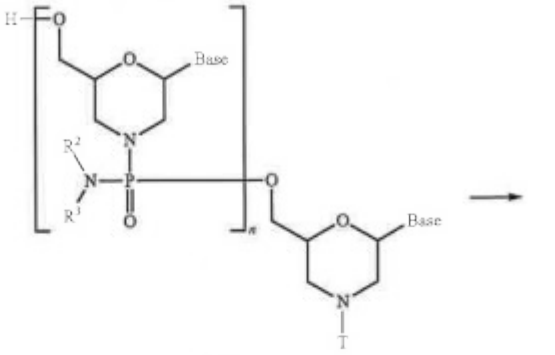
This step can be performed by reacting Compound (VII) with a deprotecting agent.

The "deprotecting agent" includes, e.g., conc. ammonia water and methylamine. The "deprotecting agent" used in this step may also be used as a dilution with, e.g., water, methanol, ethanol, isopropyl alcohol, acetonitrile, tetrahydrofuran, DMF, N,N-dimethylimidazolidone, N-methylpiperidone, or a mixture of these solvents. Among others, ethanol is preferred. The amount of the deprotecting agent used is appropriately in a range of, e.g., 1 mol equivalent to 100000 mol equivalents, and preferably in a range of 10 mol equivalents to 1000 mol equivalents, based on 1 mol of Compound (VII).

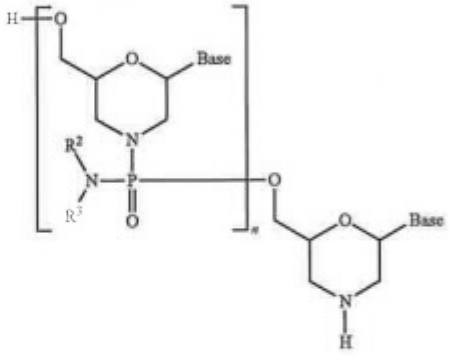
The reaction temperature is appropriately in a range of 15° C. to 75° C., preferably, in a range of 40° C. to 70° C., and more preferably, in a range of 50° C. to 60° C. The reaction time for deprotection may vary depending upon kind of Compound (VII), reaction temperature, etc., and is appropriately in a range of 10 minutes to 30 hours, preferably 30 minutes to 24 hours, and more preferably in a range of 5 hours to 20 hours.

(4) Step D:

PMO (I) is produced by reacting Compound (IX) produced in step C with an acid:



(IX)



(I)

wherein Base, n, R^2, R^3 and T have the same significance as defined above. This step can be performed by adding an acid to **Compound** (IX).

The "acid" which can be used in this step includes, for **example, trichloroacetic acid, dichloroacetic acid, acetic acid, phosphoric acid, hydrochloric acid, etc.** The **acid used is appropriately used to allow the solution** to **have a pH** range of 0.1 to 4.0, and more preferably, in a range of pH 1.0 to 3.0. The solvent is not particularly limited so long as it is inert to the reaction, and includes, for example, acetonitrile, water, or a mixture of these solvents thereof.

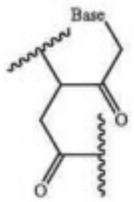
The reaction temperature is appropriately in a range of 10° C. to 50° C., preferably, in a range of 20° C. to 40° C., and more preferably, in a range of 25° C. to 35° C. The reaction time for deprotection may vary depending upon kind of Compound (IX), reaction temperature, etc., and is appropriately in a range of 0.1 minute to 5 hours, preferably 1 minute to 1 hour, and more preferably in a range of 1 minute to 30 minutes.

PMO (I) can be obtained by subjecting the reaction mixture obtained in this step to conventional means of separation and purification such as extraction, concentration, neutralization, filtration, centrifugal separation, recrystallization, reversed phase column chromatography C_8 to C_{18}, cation exchange column chromatography, anion exchange column chromatography, gel filtration column chromatography, high performance liquid chromatography, dialysis, ultrafiltration, etc., alone or in combination thereof. Thus, the desired PMO (I) can be isolated and purified (cf., e.g., WO 1991/09033).

In purification of PMO (I) using reversed phase chromatography, e.g., a solution mixture of 20 mM triethylamine/acetate buffer and acetonitrile can be used as an elution solvent.

In purification of **PMO (I) using** ion **exchange chroma**tography, e.g., a **solution mixture** of 1 M **saline solution** and 10 mM sodium hydroxide aqueous solution can be used as an elution solvent.

A peptide nucleic acid is the **oligomer** of the **present invention** having a group repres**ented by** the **following** general formula as the constituent unit:



wherein Base has the same significance as defined above.

Peptide nucleic acids can be prepared by referring to, e.g., the following literatures.

1) P. E. Nielsen, M. Egholm, R. H. Berg, O. Buchardt, Science, 254, 1497 (1991)

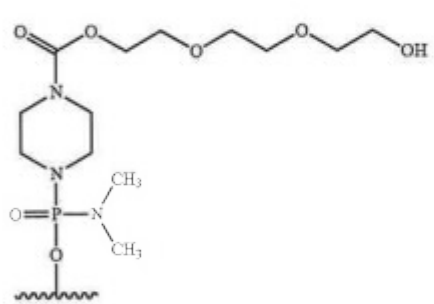
2) M. Egholm, O. Buchardt, P. E. Nielsen, R. H. Berg, Jacs., 114, 1895 (1992)

3) K. L. Dueholmn, M. Egholm, C. Behrens, L. Christensen, H. F. Hansen, T. Vulpius, K. H. Petersen, R. H. Berg, P. E. Nielsen, O. Buchardt, J. Org. Chem., 59, 5767 (1994)

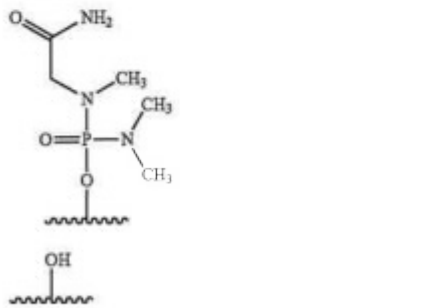
4) L. Christensen, R. Fitzpatrick, B. Gildea, K. H. Petersen, H. F. Hansen, T. Koch, M. Egholm, O. Buchardt, P. E. Nielsen, J. Coull, R. H. Berg, J. Pept. Sci., 1, 175 (1995)

5) T. Koch, H. F. Hansen, P. Andersen, T. Larsen, H. G. Batz, K. Otteson, H. Orum, J. Pept. Res., 49, 80 (1997)

In the oligomer of the present invention, the 5' end may be any of chemical structures (1) to (3) below, and preferably is (3)-OH.



(1)



(2)

(3)

Hereinafter, the groups shown by (1), (2) and (3) above are referred to as "Group (1)," "Group (2)" and "Group (3)," respectively.

2. Pharmaceutical Composition

The oligomer of the present invention causes exon 53 skipping with a higher efficiency as compared to the prior art

antisense oligomers. It is thus expected that conditions of muscular dystrophy can be relieved with high efficience by administering the pharmaceutical composition comprising the oligomer of the present invention to DMD patients. For example, when the pharmaceutical composition comprising the oligomer of the present invention is used, the same therapeutic effects can be achieved even in a smaller dose than that of the oligomers of the prior art. Accordingly, side effects can be alleviated and such is economical.

In another embodiment, the present invention provides the pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the oligomer of the present invention, a pharmaceutically acceptable salt or hydrate thereof (hereinafter referred to as "the composition of the present invention").

Examples of the pharmaceutically acceptable salt of the oligomer of the present invention contained in the composition of the present invention are alkali metal salts such as salts of sodium, potassium and lithium; alkaline earth metal salts such as salts of calcium and magnesium; metal salts such as salts of aluminum, iron, zinc, copper, nickel, cobalt, etc.; ammonium salts; organic amine salts such as salts of t-octylamine, dibenzylamine, morpholine, glucosamine, phenylglycine alkyl ester, ethylenediamine, N-methylglucamine, guanidine, diethylamine, triethylamine, dicyclohexylamine, N,N-dibenzylethylenediamine, chloroprocaine, procaine, diethanolamine, N-benzylphenethylamine, piperazine, tetramethylammonium, tris(hydroxymethyl)aminomethane; hydrohalide salts such as salts of hydrofluorates, hydrochlorides, hydrobromides and hydroiodides; inorganic acid salts such as nitrates, perchlorates, sulfates, phosphates, etc.; lower alkane sulfonates such as methanesulfonates, trifluoromethanesulfonates and ethanesulfonates; arylsulfonates such as benzenesulfonates and p-toluenesulfonates; organic acid salts such as acetates, malates, fumarates, succinates, citrates, tartarates, oxalates, maleates, etc.; and, amino acid salts such as salts of glycine, lysine, arginine, ornithine, glutamic acid and aspartic acid. These salts may be produced by known methods. Alternatively, the oligomer of the present invention contained in the composition of the present invention may be in the form of a hydrate thereof.

Administration route for the composition of the present invention is not particularly limited so long as it is pharmaceutically acceptable route for administration, and can be chosen depending upon method of treatment. In view of easiness in delivery to muscle tissues, preferred are intravenous administration, intraarterial administration, intramuscular administration, subcutaneous administration, oral administration, tissue administration, transdermal administration, etc. Also, dosage forms which are available for the composition of the present invention are not particularly limited, and include, for example, various injections, oral agents, drips, inhalations, ointments, lotions, etc.

In administration of the oligomer of the present invention to patients with muscular dystrophy, the composition of the present invention preferably contains a carrier to promote delivery of the oligomer to muscle tissues. Such a carrier is not particularly limited as far as it is pharmaceutically acceptable, and examples include cationic carriers such as cationic liposomes, cationic polymers, etc., or carriers using viral envelope. The cationic liposomes are, for example, liposomes composed of 2-O-(2-diethylaminoethyl)carabamoyl-1,3-O-dioleoylglycerol and phospholipids as the essential constituents (hereinafter referred to as "liposome A"), Oligofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectin (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine (registered trademark) (manufactured by Invitrogen Corp.), Lipofectamine 2000 (registered trademark) (manufactured by Invitrogen Corp.), DMRIE-C (registered trademark) (manufactured by Invitrogen Corp.), GeneSilencer (registered trademark) (manufactured by Gene Therapy Systems), TransMessenger (registered trademark) (manufactured by QIAGEN, Inc.), TransIT TKO (registered trademark) (manufactured by Mirus) and Nucleofector II (Lonza). Among others, liposome A is preferred. Examples of cationic polymers are JetSI (registered trademark) (manufactured by Qbiogene, Inc.) and Jet-PEI (registered trademark) (polyethylenimine, manufactured by Qbiogene, Inc.). An example of carriers using viral envelop is GenomeOne (registered trademark) (HVJ-E liposome, manufactured by Ishihara Sangyo). Alternatively, the medical devices described in Japanese Patent No. 2924179 and the cationic carriers described in Japanese Domestic Re-Publication PCT Nos. 2006/129594 and 2008/096690 may be used as well.

A concentration of the oligomer of the present invention contained in the composition of the present invention may vary depending on kind of the carrier, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the oligomer of the present invention contained in the composition of the present invention and the carrier (carrier/oligomer of the present invention) may vary depending on property of the oligomer, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20.

In addition to the oligomer of the present invention and the carrier described above, pharmaceutically acceptable additives may also be optionally formulated in the composition of the present invention. Examples of such additives are emulsification aids (e.g., fatty acids having 6 to 22 carbon atoms and their pharmaceutically acceptable salts, albumin and dextran), stabilizers (e.g., cholesterol and phosphatidic acid), isotonizing agents (e.g., sodium chloride, glucose, maltose, lactose, sucrose, trehalose), and pH controlling agents (e.g., hydrochloric acid, sulfuric acid, phosphoric acid, acetic acid, sodium hydroxide, potassium hydroxide and triethanolamine). One or more of these additives can be used. The content of the additive in the composition of the present invention is appropriately 90 wt % or less, preferably 70 wt % or less and more preferably, 50 wt % or less.

The composition of the present invention can be prepared by adding the oligomer of the present invention to a carrier dispersion and adequately stirring the mixture. Additives may be added at an appropriate step either before or after addition of the oligomer of the present invention. An aqueous solvent that can be used in adding the oligomer of the present invention is not particularly limited as far as it is pharmaceutically acceptable, and examples are injectable water or injectable distilled water, electrolyte fluid such as physiological saline, etc., and sugar fluid such as glucose fluid, maltose fluid, etc. A person skilled in the art can appropriately choose conditions for pH and temperature for such matter.

The composition of the present invention may be prepared into, e.g., a liquid form and its lyophilized preparation. The lyophilized preparation can be prepared by lyophilizing the composition of the present invention in a liquid form in a conventional manner. The lyophilization can be performed, for example, by appropriately sterilizing the composition of the present invention in a liquid form, dispensing an aliquot into a vial container, performing preliminary freezing for 2 hours at conditions of about −40 to −20° C., performing a primary drying at 0 to 10° C. under reduced pressure, and then performing a secondary drying at about 15 to 25° C. under reduced pressure. In general, the lyophilized prepa-

ration of the composition of the present invention can be obtained by replacing the content of the vial with nitrogen gas and capping.

The lyophilized preparation of the composition of the present invention can be used in general upon reconstitution by adding an optional suitable solution (reconstitution liquid) and redissolving the preparation. Such a reconstitution liquid includes injectable water, physiological saline and other infusion fluids. A volume of the reconstitution liquid may vary depending on the intended use, etc., is not particularly limited, and is suitably 0.5 to 2-fold greater than the volume prior to lyophilization or no more than 500 mL.

It is desired to control a dose of the composition of the present invention to be administered, by taking the following factors into account: the type and dosage form of the oligomer of the present invention contained; patients' conditions including age, body weight, etc.; administration route; and the characteristics and extent of the disease. A daily dose calculated as the amount of the oligomer of the present invention is generally in a range of 0.1 mg to 10 g/human, and preferably 1 mg to 1 g/human. This numerical range may vary occasionally depending on type of the target disease, administration route and target molecule. Therefore, a dose lower than the range may be sufficient in some occasion and conversely, a dose higher than the range may be required occasionally. The composition can be administered from once to several times daily or at intervals from one day to several days.

In still another embodiment of the composition of the present invention, there is provided a pharmaceutical composition comprising a vector capable of expressing the oligonucleotide of the present invention and the carrier described above. Such an expression vector may be a vector capable of expressing a plurality of the oligonucleotides of the present invention. The composition may be formulated with pharmaceutically acceptable additives as in the case with the composition of the present invention containing the oligomer of the present invention. A concentration of the expression vector contained in the composition may vary depending upon type of the career, etc., and is appropriately in a range of 0.1 nM to 100 μM, preferably in a range of 1 nM to 10 μM, and more preferably in a range of 10 nM to 1 μM. A weight ratio of the expression vector contained in the composition and the carrier (carrier/expression vector) may vary depending on property of the expression vector, type of the carrier, etc., and is appropriately in a range of 0.1 to 100, preferably in a range of 1 to 50, and more preferably in a range of 10 to 20. The content of the carrier contained in the composition is the same as in the case with the composition of the present invention containing the oligomer of the present invention, and a method for producing the same is also the same as in the case with the composition of the present invention.

Hereinafter, the present invention will be described in more detail with reference to EXAMPLES and TEST EXAMPLES below, but is not deemed to be limited thereto.

EXAMPLES

[Reference Example 1] 4-{[(2S,6R)-(4-amido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl] methoxy}-4-oxobutanoic acid laded onto aminomethyl polystyrene resin

Step 1: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl] meth oxy}-4-oxobutanoic acid

Under argon atmosphere, 22.0 g of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide and 7.04 g of 4-dimethylaminopyridine (4-DMAP) were suspended in 269 mL of dichloromethane, and 5.76 g of succinic anhydride was added to the suspension, followed by stirring at room temperature for 3 hours. To the reaction solution was added 40 mL of methanol, and the mixture was concentrated under reduced pressure. The residue was extracted using ethyl acetate and 0.5M aqueous potassium dihydrogenphosphate solution. The resulting organic layer was washed sequentially with 0.5M aqueous potassium dihydrogenphosphate solution, water and brine in the order mentioned. The resulting organic layer was dried over sodium sulfate and concentrated under reduced pressure to give 25.9 g of the product.

Step 2: Production of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1-yl)-4-tritylmorpholin-2-yl] methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

After 23.5 g of 4-{[(2S,6R)-6-(4-benzamido-2-oxopyrimidin-1(2H)-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid was dissolved in 336 mL of pyridine (dehydrated), 4.28 g of 4-DMAP and 40.3 g of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride were added to the solution. Then, 25.0 g of Aminomethyl Polystyrene Resin cross-linked with 1% DVB (manufactured by Tokyo Chemical Industry Co., Ltd., A1543) and 24 mL of triethylamine were added to the mixture, followed by shaking at room temperature for 4 days. After completion of the reaction, the resin was taken out by filtration. The resulting resin was washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure. To the resulting resin were added 150 mL of tetrahydrofuran (dehydrate), 15 mL of acetic anhydride and 15 mL of 2,6-lutidine, and the mixture was shaken at room temperature for 2 hours. The resin was taken out by filtration, washed sequentially with pyridine, methanol and dichloromethane in the order mentioned, and dried under reduced pressure to give 33.7 g of the product.

The loading amount of the product was determined by measuring UV absorbance at 409 nm of the molar amount of the trityl per g resin using a known method. The loading amount of the resin was 397.4 μmol/g.

Conditions of UV Measurement
Device: U-2910 (Hitachi, Ltd.)
Solvent: methanesulfonic acid
Wavelength: 265 nm
ε Value: 45000

[Reference Example 2] 4-Oxo-4-{[2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto 2-aminomethylpolystyrene resin

Step 1: Production of N^2-(phenoxyacetyl)guanosine

Guanosine, 100 g, was dried at 80° C. under reduced pressure for 24 hours. After 500 mL of pyridine (anhydrous) and 500 mL of dichloromethane (anhydrous) were added thereto, 401 mL of chlorotrimethylsilane was dropwise added to the mixture under an argon atmosphere at 0° C., followed by stirring at room temperature for 3 hours. The mixture was again ice-cooled and 66.3 g of phenoxyacetyl chloride was dropwise added thereto. Under ice cooling, the mixture was stirred for further 3 hours. To the reaction solution was added 500 mL of methanol, and the mixture

was stirred at room temperature overnight. The solvent was then removed by distillation under reduced pressure. To the residue was added 500 mL of methanol, and concentration under reduced pressure was performed 3 times. To the residue was added 4 L of water, and the mixture was stirred for an hour under ice cooling. The precipitates formed were taken out by filtration, washed sequentially with water and cold methanol and then dried to give 150.2 g of the objective compound (yield: 102%) (cf.: Org. Lett. (2004), Vol. 6, No. 15, 2555-2557).

Step 2: N-{9-[(2R,6S)-6-(hydroxymethyl)-4-morpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide p-toluenesulfonate

In 480 mL of methanol was suspended 30 g of the compound obtained in Step 1, and 130 mL of 2N hydrochloric acid was added to the suspension under ice cooling. Subsequently, 56.8 g of ammonium tetraborate tetrahydrate and 16.2 g of sodium periodate were added to the mixture in the order mentioned and stirred at room temperature for 3 hours. The reaction solution was ice cooled and the insoluble matters were removed by filtration, followed by washing with 100 mL of methanol. The filtrate and washing liquid were combined and the mixture was ice cooled. To the mixture was added 11.52 g of 2-picoline borane. After stirring for 20 minutes, 54.6 g of p-toluenesulfonic acid monohydrate was slowly added to the mixture, followed by stirring at 4° C. overnight. The precipitates were taken out by filtration and washed with 500 mL of cold methanol and dried to give 17.7 g of the objective compound (yield: 43.3%).

^{1}H NMR (8, DMSO-d6): 9.9-9.2 (2H, br), 8.35 (1H, s), 7.55 (2H, m), 7.35 (2H, m), 7.10 (2H, d, J=7.82 Hz), 7.00 (3H, m), 5.95 (1H, dd, J=10.64, 2.42 Hz), 4.85 (2H, s), 4.00 (1H, m), 3.90-3.60 (2H, m), 3.50-3.20 (5H, m), 2.90 (1H, m), 2.25 (3H, s)

Step 3: Production of N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide

In 30 mL of dichloromethane was suspended 2.0 g of the compound obtained in Step 2, and 13.9 g of triethylamine and 18.3 g of trityl chloride were added to the suspension under ice cooling. The mixture was stirred at room temperature for an hour. The reaction solution was washed with saturated sodium bicarbonate aqueous solution and then with water, and dried. The organic layer was concentrated under reduced pressure. To the residue was added 40 mL of 0.2M sodium citrate buffer (pH 3)/methanol (1:4 (v/v)), and the mixture was stirred. Subsequently, 40 mL of water was added and the mixture was stirred for an hour under ice cooling. The mixture was taken out by filtration, washed with cold methanol and dried to give 1.84 g of the objective compound (yield: 82.0%).

Step 4: Production of 4-oxo-4-{[(2S,6R)-6-(6-oxo-2-[2-phenoxyacetamido]-1H-purin-9-yl)-4-tritylmorpholin-2-yl]methoxy}butanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that N-{9-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-6-oxo-6,9-dihydro-1H-purin-2-yl}-2-phenoxyacetamide was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[Reference Example 3] 4-{[(2S,6R)-6-(5-Methyl-2,4-dioxo-3,4-dihydropyrimidin-1-yl)-4-tritylmorpholin-2-yl]methoxy}-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-5-methylpyrimidine-2,4(1H,3H)-dione was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide used in Step 1 of REFERENCE EXAMPLE 1.

[Reference Example 4] 1,12-Dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin

The title compound was produced in a manner similar to REFERENCE EXAMPLE 1, except that 2-[2-(2-hydroxyethoxyl)ethoxy]ethyl 4-tritylpiperazine-1-carboxylic acid (the compound described in WO 2009/064471) was used in this step, instead of N-{1-[(2R,6S)-6-(hydroxymethyl)-4-tritylmorpholin-2-yl]-2-oxo-1,2-dihydropyrimidin-4-yl}benzamide.

According to the descriptions in EXAMPLES 1 to 12 and REFERENCE EXAMPLES 1 to 3 below, various types of PMO shown by PMO Nos. 1-11 and 13-16 in TABLE 2 were synthesized. The PMO synthesized was dissolved in injectable water (manufactured by Otsuka Pharmaceutical Factory, Inc.). PMO No. 12 was purchased from Gene Tools, LLC.

TABLE 2

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
1	31-55	5' end: group (3)	SEQ ID NO: 4
2	32-53	5' end: group (3)	SEQ ID NO: 8
3	32-56	5' end: group (3)	SEQ ID NO: 11
4	33-54	5' end: group (3)	SEQ ID NO: 15
5	34-58	5' end: group (3)	SEQ ID NO: 25
6	36-53	5' end: group (3)	SEQ ID NO: 32
7	36-55	5' end: group (3)	SEQ ID NO: 34
8	36-56	5' end: group (3)	SEQ ID NO: 35
9	36-57	5' end: group (3)	SEQ ID NO: 36

TABLE 2-continued

PMO No.	Target sequence in exon 53	Note	SEQ ID NO:
10	33-57	5' end: group (3)	SEQ ID NO: 18
11	39-69	Sequence corresponding to H53A(+39 + 69) (cf. Table 1) in Non-Patent Document 3, 5' end: group (3)	SEQ ID NO: 38
12	30-59	Sequence corresponding to h53A30/1 (cf. Table 1) in Non-Patent Document 5, 5' end: group (2)	SEQ ID NO: 39
13	32-56	5' end: group (1)	SEQ ID NO: 11
14	36-56	5' end: group (1)	SEQ ID NO: 35
15	30-59	Sequence corresponding to h53A30/1 cf. Table 1) in Non-Patent Document 5 5' end: group (3)	SEQ ID NO: 39
16	23-47	Sequence corresponding to SEQ ID NO: 429 described in Patent Document 4, 5' end: group (3)	SEQ ID NO: 47

Example 1

PMO No. 8

4-{[(2S,6R)-6-(4-**Benzamido-2-oxopyrimidin**-1(2H)-yl)-4-tritylmorpholin-2-**yl]methoxy**}-**4-oxobutanoic** acid, loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 1), 2 g (800 μmol) was transferred to a reaction vessel, and 30 mL of dichloromethane was added thereto. The mixture was allowed to stand for 30 minutes. After the mixture was further washed twice with 30 mL of dichloromethane, the following synthesis cycle was started. The desired morpholino monomer compound was added in each cycle to give the nucleotide sequence of the title compound.

TABLE 3

Step	Reagent	Volume (mL)	Time (min)
1	deblocking solution	30	2.0
2	deblocking solution	30	2.0
3	deblocking solution	30	2.0
4	deblocking solution	30	2.0
5	deblocking solution	30	2.0
6	deblocking solution	30	2.0
7	neutralizing solution	30	1.5
8	neutralizing solution	30	1.5
9	neutralizing solution	30	1.5
10	neutralizing solution	30	1.5
11	neutralizing solution	30	1.5
12	neutralizing solution	30	1.5
13	dichloromethane	30	0.5
14	dichloromethane	30	0.5
15	dichloromethane	30	0.5
16	coupling solution B	20	0.5
17	coupling solution A	6-11	90.0
18	dichloromethane	30	0.5
19	dichloromethane	30	0.5
20	dichloromethane	30	0.5
21	capping solution	30	3.0
22	capping solution	30	3.0
23	dichloromethane	30	0.5
24	dichloromethane	30	0.5
25	dichloromethane	30	0.5

The deblocking solution used was a solution obtained by dissolving a mixture of trifluoroacetic acid (2 equivalents) and triethylamine (1 equivalent) in a dichloromethane solution containing 1% (v/v) ethanol and 10% (v/v) 2,2,2-trifluoroethanol to be 3% (w/v). The neutralizing solution used was a solution obtained by dissolving N,N-diisopropylethylamine in a dichloromethane solution containing 25% (v/v) 2-propanol to be 5% (v/v). The coupling solution A used was a solution obtained by dissolving the morpholino monomer compound in 1,3-dimethyl-2-imidazolidinone containing 10% (v/v) N,N-diisopropylethylamine to be 0.15M. The coupling solution B used was a solution obtained by dissolving N,N-diisopropylethylamine in 1,3-dimethyl-2-imidazolidinone to be 10% (v/v). The capping solution used was a solution obtained by dissolving 20% (v/v) acetic anhydride and 30% (v/v) 2,6-lutidine in dichloromethane.

The aminomethyl polystyrene resin loaded with the PMO synthesized above was recovered from the reaction vessel and dried at room temperature for at least 2 hours under reduced pressure. The dried PMO loaded onto aminomethyl polystyrene resin was charged in a reaction vessel, and 200 mL of 28% ammonia water-ethanol (1/4) was added thereto. The mixture was stirred at 55° C. for 15 hours. The aminomethyl polystyrene resin was separated by filtration and washed with 50 mL of water-ethanol (1/4). The resulting filtrate was concentrated under reduced pressure. The resulting residue was dis**solved in 100 mL of a solvent mixture** of 20 mM acetic acid-**triethylamine buffer (TEAA buffer)** and acetonitrile (4/1) and filtered through a membrane filter. The filtrate obtained was purified by reversed phase HPLC. The conditions used are as follows.

TABLE 4

Column	XTera MS18 (Waters, ϕ50 × 100 mm, 1CV = 200 mL)
Flow rate	60 mL/min
Column temperature	room temperature
Solution A	20 mM TEAA buffer
Solution B	CH_3CN
Gradient	(B) conc. 20 → 50%/9CV

Each fraction was **analyzed and the product was recovered in 100 mL of acetonitrile-water (1/1), to which 200 mL** of ethanol was added. **The mixture was concentrated under** reduced pressure. Further drying under reduced pressure gave a white solid. To the resulting solid was added 300 mL of 10 mM phosphoric acid aqueous solution to suspend the solid. To the suspension was added 10 mL of 2M phosphoric acid aqueous solution, and the mixture was stirred for 15 minutes. Furthermore, 15 mL of 2M sodium hydrate aqueous solution was added for neutralization. Then, 15 mL of 2M sodium hydroxide aqueous solution was added to make the mixture alkaline, followed by filtration through a membrane filter (0.45 μm). The mixture was thoroughly washed

with 100 mL of 10 mM sodium hydroxide aqueous solution to give the product as an aqueous solution.

The resulting aqueous solution containing the product was purified by an anionic exchange resin column. The conditions used are as follows.

TABLE 5

Column	Source 30Q (GE Healthcare,ϕ 40 × 150 mm, 1CV = 200 mL)
Flow rate	80 mL/min
Column temp.	room temperature
Solution A	10 mM sodium hydroxide aqueous solution
Solution B	10 mM sodium hydroxide aqueous solution, 1M sodium chloride aqueous solution
Gradient	(B) conc. 5 → 35%/15CV

Each fraction was analyzed (on HPLC) and the product was obtained as an aqueous solution. To the resulting aqueous solution was added 225 mL of 0.1M phosphate buffer (pH 6.0) for neutralization. The mixture was filtered through a membrane filter (0.45 μm). Next, ultrafiltration was performed under the conditions

TABLE 6

Filter	PELLICON2 MINI FILTER PLBC 3K Regenerated Cellulose, Screen Type C
Size	0.1 m^2

The filtrate was concentrated to give approximately 250 mL of an aqueous solution. The resulting aqueous solution was filtered through a membrane filter (0.45 μm). The aqueous solution obtained was freeze-dried to give 1.5 g of the objective compound as a white cotton-like solid.

ESI-TOF-MS Calcd.: 6924.82.
Found: 6923.54.

Example 2

PMO. No. 1

The title compound was produced in accordance with the procedure of EXAMPLE 1.

MALDI-TOF-MS Calcd.: 8291.96.
Found: 8296.24.

Example 3

PMO. No. 2

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 7310.13.
Found: 7309.23.

Example 4

PMO. No. 3

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8270.94.
Found: 8270.55.

Example 5

PMO. No. 4

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-(2H)-yl)-4-trityl-morpholin-2-yl)methoxy)-4-oxobutanoic acid (REFERENCE EXAMPLE 3) loaded onto aminomethyl polystyrene resin was used as the starting material.

ESI-TOF-MS Calcd.: 7310.13.
Found: 7310.17.

Example 6

PMO. No. 5

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-(((2S,6R)-6-(5-methyl-2,4-dioxo-3,4-dihydropyrimidin-1(2H)-yl)-4-trityl-morpholin-2-yl)methoxy)-4-oxobutanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 3) was used as the starting material.

ESI-TOF-MS Calcd.: 8270.94.
Found: 8270.20.

Example 7

PMO. No. 6

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 5964.01.
Found: 5963.68.

Example 8

PMO. No. 7

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 6609.55.
Found: 6608.85.

Example 9

PMO. No. 9

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 7280.11.
Found: 7279.4.

Example 10

PMO. No. 10

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 4-oxo-4-(((2S,6R)-

6-(6-oxo-2-(2-phenoxyacetamido)-1H-purin-9(6H)-yl)-4-tritylmorpholin-2-yl)methoxy)butanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 2) was used as the starting material.

ESI-TOF-MS Calcd.: 8295.95.

Found: 8295.91.

Example 11

PMO. No. 13

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-15-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 7276.15.

Found: 7276.69.

Example 12

PMO. No. 14

The title compound was produced in accordance with the procedure of EXAMPLE 1, except that 1,12-dioxo-1-(4-tritylpiperazin-1-yl)-2,5,8,11-tetraoxa-5-pentadecanoic acid loaded onto aminomethyl polystyrene resin (REFERENCE EXAMPLE 4) was used as the starting material.

ESI-TOF-MS Calcd.: 8622.27.

Found: 8622.29.

Comparative Example 1

PMO. No. 11

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 10274.63.

Found: 10273.71.

Comparative Example 2

PMO. No. 15

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 9941.33.

Found: 9940.77.

Comparative Example 3

PMO. No. 16

The title compound was produced in accordance with the procedure of EXAMPLE 1.

ESI-TOF-MS Calcd.: 8238.94.

Found: 8238.69.

Test Example 1

In Vitro Assay

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 10 μM of the oligomers PMO Nos. 1 to 8 of the present invention and the antisense oligomer PMO No. 11 were transfected with 4×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer: (SEQ ID NO: 40)
5'-AGGATTTGGAACAGAGGCGTC-3'

Reverse primer: (SEQ ID NO: 41)
5'-GTCTGCCACTGGCGGAGGTC-3'

Next, a nested PCR was performed with the product amplified by RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer: (SEQ ID NO: 42)
5'-CATCAAGCAGAAGGCAACAA-3'

Reverse primer: (SEQ ID NO: 43)
5'-GAAGTTTCAGGGCCAAGTCA-3'

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

Skipping efficiency (%)=$A/(A+B)\times100$

Experimental Results

The results are shown in FIG. 1. This experiment revealed that the oligomers PMO Nos. 1 to 8 of the present invention

caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomer PMO No. 11. In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than four times higher exon skipping efficiency than that of the antisense oligomer PMO No. 11.

Test Example 2

In Vitro Assay Using Human Fibroblasts

Human myoD gene (SEQ ID NO: 44) was introduced into TIG-119 cells (human normal tissue-derived fibroblasts, National Institute of Biomedical Innovation) or 5017 cells (human DMD patient-derived fibroblasts, Coriell Institute for Medical Research) using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12 to 14 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and the morpholino oligomer was added thereto in a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEX51F and hEX55R.

hEX51F:
(SEQ ID NO: 45)
5'-CGGGCTTGGACAGAACTTAC-3'

hEx55R:
(SEQ ID NO: 46)
5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

Skipping efficiency (%)=$A/(A+B)\times100$

Experimental Results

The results are shown in FIGS. **2** and **3**. This experiment revealed that in TIG-119 cells, the oligomers PMO Nos. 3, 8 and 9 of the present invention (FIG. **2**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **2**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than twice higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **2**).

Furthermore, this experiment revealed that the oligomers PMO Nos. 3 and 8 to 10 of the present invention (FIG. **3**) all caused exon 53 skipping with a higher efficiency than the antisense oligomer PMO No. 12 (FIG. **3**). In particular, the oligomers PMO Nos. 3 and 8 of the present invention exhibited more than seven times higher exon skipping efficiency than that of the antisense oligomer PMO No. 12 (FIG. **3**).

Test Example 3

In Vitro Assay Using Human Fibroblasts

The skin fibroblast cell line (fibroblasts from human DMD patient (exons 45-52 or exons 48-52)) was established by biopsy from the medial left upper arm of DMD patient with deletion of exons 45-52 or DMD patient with deletion of exons 48-52. Human myoD gene (SEQ ID NO: 44) was introduced into the fibroblast cells using a ZsGreen1 coexpression retroviral vector.

After incubation for 4 to 5 days, ZsGreen-positive MyoD-transformed fibroblasts were collected by FACS and plated at $5\times10^4/cm^2$ into a 12-well plate. As a growth medium, there was used 1 mL of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) (Invitrogen Corp.) containing 10% FCS and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich, Inc.).

The medium was replaced 24 hours later by a differentiation medium (DMEM/F-12 containing 2% equine serum (Invitrogen Corp.), 1% P/S and ITS Liquid Media Supplement (Sigma, Inc.)). The medium was exchanged every 2 to 3 days and incubation was continued for 12, 14 or 20 days to differentiate into myotubes.

Subsequently, the differentiation medium was replaced by a differentiation medium containing 6 μM Endo-Porter (Gene Tools), and a morpholino oligomer was added thereto at a final concentration of 10 μM. After incubation for 48 hours, total RNA was extracted from the cells using a TRIzol (manufactured by Invitrogen Corp.). RT-PCR was performed with 50 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit. A reaction solution was prepared in accordance with the protocol attached to the kit. An iCycler (manufactured by Bio-Rad) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 1 mins; 60° C., 1 mins; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 7 mins: final extension

The primers used were hEx44F and h55R.

hEx44F:
(SEQ ID NO: 48)
5'-TGTTGACAAATGGCGGCGT-3'

hEx55R:
(SEQ ID NO: 46)
5'-TCCTTACGGGTAGCATCCTG-3'

The reaction product of RT-PCR above was separated by 2% agarose gel electrophoresis and gel images were captured with a GeneFlash (Syngene). The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured using an Image J (manufactured by National Institutes of Health). Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation.

$$\text{Skipping efficiency (\%)} = A/(A+B) \times 100$$

Experimental Results

The results are shown in FIGS. **4** and **5**. This experiment revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with an efficiency as high as more than 80% in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**) or deletion of exons 48-52 (FIG. **5**). Also, the oligomers PMO Nos. 3 and 8 of the present invention were found to cause exon 53 skipping with a higher efficiency than that of the antisense oligomer PMO No. 15 in the cells from DMD patient with deletion of exons 45-52 (FIG. **4**).

Test Example 4

Western Blotting

The oligomer PMO No. 8 of the present invention was added to the cells at a concentration of 10 μM, and proteins were extracted from the cells after 72 hours using a RIPA buffer (manufactured by Thermo Fisher Scientific) containing Complete Mini (manufactured by Roche Applied Science) and quantified using a BCA protein assay kit (manufactured by Thermo Fisher Scientific). The proteins were electrophoresed in NuPAGE Novex Tris-Acetate Gel 3-8% (manufactured by Invitrogen) at 150V for 75 minutes and transferred onto a PVDF membrane (manufactured by Millipore) using a semi-dry blotter. The PVDF membrane was blocked with a 5% ECL Blocking agent (manufactured by GE Healthcare) and the membrane was then incubated in a solution of anti-dystrophin antibody (manufactured by NCL-Dys1, Novocastra). After further incubation in a solution of peroxidase-conjugated goat-antimouse IgG (Model No. 170-6516, Bio-Rad), the membrane was stained with ECL Plus Western blotting system (manufactured by GE Healthcare).

Immunostaining

The oligomer PMO No. 3 or 8 of the present invention was added to the cells. The cells after 72 hours were fixed in 3% paraformaldehyde for 10 minutes, followed by incubation in 10% Triton-X for 10 minutes. After blocking in 10% goat serum-containing PBS, the membrane was incubated in a solution of anti-dystrophin antibody (NCL-Dys1, Novocastra). The membrane was further incubated in a solution of anti-mouse IgG antibody (manufactured by Invitrogen). The membrane was mounted with Pro Long Gold Antifade reagent (manufactured by Invitrogen) and observed with a fluorescence microscope.

Experimental Results

The results are shown in FIGS. **6** and **7**. In this experiment it was confirmed by western blotting (FIG. **6**) and immunostaining (FIG. **7**) that the oligomers PMO Nos. 3 and 8 of the present invention induced expression of the dystrophin protein.

Test Example 5

In Vitro Assay Using Human Fibroblasts

The experiment was performed as in TEST EXAMPLE 3.

Experimental Results

The results are shown in FIG. **8**. This experiment revealed that in the cells from DMD patients with deletion of exons 45-52, the oligomers PMO Nos. 3 to 8 of the present invention caused exon 53 skipping with a higher efficiency than the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **8**).

Test Example 6

In Vitro Assay

Experiments were performed using the antisense oligomers of 2'-O-methoxy-phosphorothioates (2'-OMe-S-RNA) shown by SEQ ID NO: 49 to SEQ ID NO: 123. Various antisense oligomers used for the assay were purchased from Japan Bio Services. The sequences of various antisense oligomers are given below.

TABLE 7

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_39-69	CAUUCAACUGGUGCCUCCGGUUCUGAAGGUG	49
H53_1-25	UCCCACUGAUUCUGAAUUCUUUCAA	50
H53_6-30	CUUCAUCCCACUGAUUCUGAAUUCU	51
H53_11-35	UUGUACUUCAUCCCACUGAUUCUGA	52
H53_16-40	UGUUCUUGUACUUCAUCCCACUGAU	53
H53_21-45	GAAGGUGUUCUUGUACUUCAUCCCA	54
H53_26-50	GUUCUGAAGGUGUUCUUGUACUUCA	55
H53_31-55	CUCCGGUUCUGAAGGUGUUCUUGUA	56
H53_36-60	GUUGCCUCCGGUUCUGAAGGUGUUC	57
H53_41-65	CAACUGUUGCCUCCGGUUCUGAAGG	58
H53_46-70	UCAUUCAACUGUUGCCUCCGGUUCU	59
H53_51-75	ACAUUUCAUUCAACUGUUGCCUCCG	60
H53_56-80	CUUUAACAUUUCAUUCAACUGUUGC	61
H53_61-85	GAAUCCUUUAACAUUUCAUUCAACU	62
H53_66-90	GUGUUGAAUCCUUUAACAUUUCAUU	63
H53_71-95	CCAUUGUGUUGAAUCCUUUAACAUU	64
H53_76-100	UCCAGCCAUUGUGUUGAAUCCUUUA	65
H53_81-105	UAGCUUCCAGCCAUUGUGUUGAAUC	66
H53_86-110	UUCCUUAGCUUCCAGCCAUUGUGUU	67
H53_91-115	GCUUCUUCCUUAGCUUCCAGCCAUU	68

TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_96-120	GCUCAGCUUCUUCCUUAGCUUCCAG	69
H53_101-125	GACCUGCUCAGCUUCUUCCUUAGCU	70
H53_106-130	CCUAAGACCUGCUCAGCUUCUUCCU	71
H53_111-135	CCUGUCCUAAGACCUGCUCAGCUUC	72
H53_116-140	UCUGGCCUGUCCUAAGACCUGCUCA	73
H53_121-145	UUGGCUCUGGCCUGUCCUAAGACCU	74
H53_126-150	CAAGCUUGGCUCUGGCCUGUCCUAA	75
H53_131-155	UGACUCAAGCUUGGCUCUGGCCUGU	76
H53_136-160	UUCCAUGACUCAAGCUUGGCUCUGG	77
H53_141-165	CCUCCUUCCAUGACUCAAGCUUGGC	78
H53_146-170	GGGACCCUCCUUCCAUGACUCAAGC	79
H53_151-175	GUAUAGGGACCCUCCUUCCAUGACU	80
H53_156-180	CUACUGUAUAGGGACCCUCCUUCCA	81
H53_161-185	UGCAUCUACUGUAUAGGGACCCUCC	82
H53_166-190	UGGAUUGCAUCUACUGUAUAGGGAC	83
H53_171-195	UCUUUUGGAUUGCAUCUACUGUAUA	84
H53_176-200	GAUUUUCUUUUGGAUUGCAUCUACU	85
H53_181-205	UCUGUGAUUUUCUUUUGGAUUGCAU	86
H53_186-210	UGGUUUCUGUGAUUUUCUUUUGGAU	87
H53_84-108	CCUUAGCUUCCAGCCAUUGUGUUGA	88
H53_88-112	UCUUCCUUAGUUCCAGCCAUUGUG	89
H53_119-143	GGCUCUGGCCUGUCCUAAGACCUGC	90
H53_124-148	AGCUUGGCUCUGGCCUGUCCUAAGA	91
H53_128-152	CUCAAGCUUGGCUCUGGCCUGUCCU	92
H53_144-168	GACCCUCCUUCCAUGACUCAAGCUU	93
H53_149-173	AUAGGGACCCUCCUUCCAUGACUCA	94
H53_153-177	CUGUAUAGGGACCCUCCUUCCAUCGA	95
H53_179-203	UGUGAUUUUCUUUUGGAUUGCAUCU	96
H53_184-208	GUUUCUGUGAUUUUCUUUUGGAUUG	97
H53_188-212	CUUGGUUUCUGUGAUUUUCUUUUGG	98
H53_29-53	CCGGUUCUGAAGGUGUUCUUGUACU	99
H53_30-54	UCCGGUUCUGAAGGUGUUCUUGUAC	100
H53_32-56	CCUCCGGUUCUGAAGGUGUUCUUGU	101
H53_33-57	GCCUCCGGUUCUGAAGGUGUUCUUG	102
H53_34-58	UGCCUCCGGUUCUGAAGGUGUUCUU	103
H53_35-59	UUGCCUCCGGUUCUGAAGGUGUUCU	104
H53_37-61	UGUUGCCUCCGGUUCUGAAGGUGUU	105
H53_38-62	CUGUUGCCUCCGGUUCUGAAGGUGU	106
H53_39-63	ACUGUUGCCUCCGGUUCUGAAGGUG	107

TABLE 7-continued

Antisense oligomer	Nucleotide sequence	SEQ ID NO:
H53_40-64	AACUGUUGCCUCCGGUUCUGAAGGU	108
H53_32-61	UGUUGCCUCCGGUUCUGAAGGUGUUCUUGU	109
H53_32-51	GGUUCUGAAGGUGUUCUUGU	110
H53_35-54	UCCGGUUCUGAAGGUGUUCU	111
H53_37-56	CCUCCGGUUCUGAAGGUGUU	112
H53_40-59	UUGCCUCCGGUUCUGAAGGU	113
H53_42-61	UGUUGCCUCCGGUUCUGAAG	114
H53_32-49	UUCUGAAGGUGUUCUUGU	115
H53_35-52	CGGUUCUGAAGGUGUUCU	116
H53_38-55	CUCCGGUUCUGAAGGUGU	117
H53_41-58	UGCCUCCGGUUCUGAAGG	118
H53_44-61	UGUUGCCUCCGGUUCUGA	119
H53_35-49	UUCUGAAGGUGUUCU	120
H53_40-54	UCCGGUUCUGAAGGU	121
H53_45-59	UUGCCUCCGGUUCUG	122
H53_45-62	CUGUUGCCUCCGGUUCUG	123

RD cells (human rhabdomyosarcomna cell line) were plated at 3×10^5 in a 6-well plate and cultured in 2 ml of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2 overnight. Complexes of various antisense oligomers (Japan Bio Services) (1 μM) for exon 53 skipping and Lipofectamine 2000 (manufactured by Invitrogen Corp.) were prepared and 200 μl was added to RD cells where 1.8 mL of the medium was exchanged, to reach the final concentration of 100 nM.

After completion of the addition, the cells were cultured overnight. The cells were washed twice with PBS (manufactured by Nissui, hereafter the same) and then 500 μl of ISOGEN (manufactured by Nippon Gene) were added to the cells. After the cells were allowed to stand at room temperature for a few minutes for cell lysis, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a Titan One Tube RT-PCR Kit (manufactured by Roche). A reaction solution was prepared in accordance with the protocol attached to the kit. A PTC-100 (manufactured by MJ Research) was used as a thermal cycler. The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

94° C., 2 mins: thermal denaturation

[94° C., 10 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer:
(SEQ ID NO: 42)
5'CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
(SEQ ID NO: 43)
5'-GAAGTTTCAGGGCCAAGTCA-3'

Subsequently, a nested PCR was performed with the amplified product of RT-PCR above using a Taq DNA Polymerase (manufactured by Roche). The PCR program used is as follows.

94° C., 2 mins: thermal denaturation

[94° C., 15 seconds; 58° C., 30 seconds; 68° C., 45 seconds]×30 cycles: PCR amplification

68° C., 7 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for the nested PCR above are given below.

Forward primer:
(SEQ. ID NO: 40)
5'-AGGATTTGGAACAGAGGCGTC-3'

Reverse primer:
(SEQ ID NO: 41)
5'-GTCTGCCACTGGCGGAGGTC-3'

The reaction product, 1 μl, of the nested PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **9** to **17**. These experiments revealed that, when the antisense oligomers were designed at exons 31-61 from the 5' end of exon 53 in the human dystrophin gene, exon 53 skipping could be caused with a high efficiency.

Test Example 7

Using an Amaxa Cell Line Nucleofector Kit L on Nucleofector II (Lonza), 0.3 to 30 μM of the antisense oligomers were transfected with 3.5×10^5 of RD cells (human rhabdomyosarcoma cell line). The Program T-030 was used.

After the transfection, the cells were cultured overnight in 2 mL of Eagle's minimal essential medium (EMEM) (manufactured by Sigma, Inc., hereinafter the same) containing 10% fetal calf serum (FCS) (manufactured by Invitrogen Corp.) under conditions of 37° C. and 5% CO_2. The cells were washed twice with PBS (manufactured by Nissui, hereinafter the same) and 500 μl of ISOGEN (manufactured by Nippon Gene) was then added to the cells. After the cells were allowed to stand at room temperature for a few minutes to lyse the cells, the lysate was collected in an Eppendorf tube. The total RNA was extracted according to the protocol attached to ISOGEN. The concentration of the total RNA extracted was determined using a NanoDrop ND-1000 (manufactured by LMS).

One-Step RT-PCR was performed with 400 ng of the extracted total RNA using a QIAGEN OneStep RT-PCR Kit (manufactured by Qiagen, Inc.). A reaction solution was prepared in accordance with the protocol attached to the kit. The thermal cycler used was a PTC-100 (manufactured by MJ Research). The RT-PCR program used is as follows.

50° C., 30 mins: reverse transcription

95° C., 15 mins: thermal denaturation

[94° C., 30 seconds; 60° C., 30 seconds; 72° C., 1 mins]×35 cycles: PCR amplification

72° C., 10 mins: final extension

The nucleotide sequences of the forward primer and reverse primer used for RT-PCR are given below.

Forward primer:
(SEQ. ID NO: 42)
5'-CATCAAGCAGAAGGCAACAA-3'

Reverse primer:
(SEQ. ID NO: 43)
5'-GAAGTTTCAGGCCAAGTCA-3'

The reaction product, 1 μl, of the PCR above was analyzed using a Bioanalyzer (manufactured by Agilent Technologies, Inc.).

The polynucleotide level "A" of the band with exon 53 skipping and the polynucleotide level "B" of the band without exon 53 skipping were measured. Based on these measurement values of "A" and "B," the skipping efficiency was determined by the following equation:

$$\text{Skipping efficiency (\%)}=A/(A+B)\times 100$$

Experimental Results

The results are shown in FIGS. **18** and **19**. These experiments revealed that the oligomer PMO No. 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the antisense oligomers PMO Nos. 15 and 16 (FIG. **18**). It was also revealed that the oligomers PMO Nos. 3 and 8 of the present invention caused exon 53 skipping with a markedly high efficiency as compared to the oligomers PMO Nos. 13 and 14 of the present invention (FIG. **19**). These results showed that the sequences with —OH group at the 5' end provide a higher skipping efficiency even in the same sequences.

INDUSTRIAL APPLICABILITY

Experimental results in TEST EXAMPLES demonstrate that the oligomers of the present invention (PMO Nos. 1 to 10) all caused exon 53 skipping with a markedly high efficiency under all cell environments, as compared to the oligomers (PMO Nos. 11, 12, 15 and 16) in accordance with the prior art. The 5017 cells used in TEST EXAMPLE 2 are the cells isolated from DMD patients, and the fibroblasts used in TEST EXAMPLES 3 and 5 are exon 53 skipping target cells from DMD patients. Particularly in TEST EXAMPLES 3 and 5, the oligomers of the present invention show the exon 53 skipping efficiency of 90% or higher in the cells from DMD patients that are the target for exon 53 skipping. Consequently, the oligomers of the present invention can induce exon 53 skipping with a high efficiency, when DMD patients are administered.

Therefore, the oligomers of the present invention are extremely useful for the treatment of DMD.

Sequence Listing Free Text

SEQ ID NO: 2: synthetic nucleic acid
SEQ ID NO: 3: synthetic nucleic acid
SEQ ID NO: 4: synthetic nucleic acid
SEQ ID NO: 5: synthetic nucleic acid
SEQ ID NO: 6: synthetic nucleic acid
SEQ ID NO: 7: synthetic nucleic acid
SEQ ID NO: 8: synthetic nucleic acid

SEQ ID NO: 9: synthetic nucleic acid
SEQ ID NO: 10: synthetic nucleic acid
SEQ ID NO: 11: synthetic nucleic acid
SEQ ID NO: 12: synthetic nucleic acid
SEQ ID NO: 13: synthetic nucleic acid
SEQ ID NO: 14: synthetic nucleic acid
SEQ ID NO: 15: synthetic nucleic acid
SEQ ID NO: 16: synthetic nucleic acid
SEQ ID NO: 17: synthetic nucleic acid
SEQ ID NO: 18: synthetic nucleic acid
SEQ ID NO: 19: synthetic nucleic acid
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SEQ ID NO: 112: synthetic nucleic acid
SEQ ID NO: 113: synthetic nucleic acid
SEQ ID NO: 114: synthetic nucleic acid
SEQ ID NO: 115: synthetic nucleic acid
SEQ ID NO: 116: synthetic nucleic acid
SEQ ID NO: 117: synthetic nucleic acid
SEQ ID NO: 118: synthetic nucleic acid
SEQ ID NO: 119: synthetic nucleic acid
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SEQ ID NO: 122: synthetic nucleic acid
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Sequence Listing

SEQUENCE LISTING

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aggtcttagg acaggccaga gccaagcttg agtcatggaa ggagggtccc tatacagtag 180

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gcctccggtt ctgaaggtgt tctt 24

<210> SEQ ID NO 25
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<220> FEATURE:
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tccggttctg aaggtgttct 20

<210> SEQ ID NO 28
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ctccggttct gaaggtgttc t 21

<210> SEQ ID NO 29
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<400> SEQUENCE: 29

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gcctccggtt ctgaaggtgt tct 23

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<211> LENGTH: 24
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<400> SEQUENCE: 32

ccggttctga aggtgttc 18

<210> SEQ ID NO 33
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<400> SEQUENCE: 33

tccggttctg aaggtgttc 19

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<400> SEQUENCE: 34

ctccggttct gaaggtgttc 20

<210> SEQ ID NO 35
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gcctccggtt ctgaaggtgt tc 22

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tgcctccggt tctgaaggtg ttc 23

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<400> SEQUENCE: 38

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<210> SEQ ID NO 39
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<212> TYPE: DNA
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aggatttgga acagaggcgt c 21

<210> SEQ ID NO 41
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<400> SEQUENCE: 41

gtctgccact ggcggaggtc 20

<210> SEQ ID NO 42
<211> LENGTH: 20
<212> TYPE: DNA
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<400> SEQUENCE: 42

catcaagcag aaggcaacaa 20

<210> SEQ ID NO 43
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<400> SEQUENCE: 43

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<210> SEQ ID NO 44
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<212> TYPE: DNA

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<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 44

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cgcttcttcg aagacctgga cccgcgcctg atgcacgtgg gcgcgctcct gaaaccccgaa 180

gagcactcgc acttccccgc ggcggtgcac ccggcccccgg gcgcacgtga ggacgagcat 240

gtgcgcgcgc ccagcgggca ccaccaggcg ggccgctgcc tactgtgggc ctgcaaggcg 300

tgcaagcgca agaccaccaa cgccgaccgc cgcaaggccg ccaccatgcg cgagcggcgc 360

cgcctgagca aagtaaatga ggcctttgag acactcaagc gctgcacgtc gagcaatcca 420

aaccagcggt tgcccaaggt ggagatcctg cgcaacgcca tccgctatat cgagggcctg 480

caggctctgc tgcgcgacca ggacgccgcg ccccctggcg ccgcagccgc cttctatgcg 540

ccgggcccgc tgccccgggg ccgcggcggc gagcactaca gcggcgactc cgacgcgtcc 600

agcccgcgct ccaactgctc cgacggcatg atggactaca gcggcccccc gagcggcgcc 660

cggcggcgga actgctacga aggcgcctac tacaacgagg cgcccagcga acccaggccc 720

gggaagagtg cggcggtgtc gagcctagac tgcctgtcca gcatcgtgga gcgcatctcc 780

accgagagcc ctgcggcgcc cgcccctcctg ctggcggacg tgccttctga gtcgcctccg 840

cgcaggcaag aggctgccgc ccccagcgag ggagagagca gcggcgaccc cacccagtca 900

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tga 963

<210> SEQ ID NO 45
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<212> TYPE: DNA
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<400> SEQUENCE: 45

cgggcttgga cagaacttac 20

<210> SEQ ID NO 46
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 46

tccttacggg tagcatcctg 20

<210> SEQ ID NO 47
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 47

ctgaaggtgt tcttgtactt catcc 25

<210> SEQ ID NO 48
<211> LENGTH: 19
<212> TYPE: DNA
<213> ORGANISM: Artificial

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<220> FEATURE:
<223> OTHER INFORMATION: Synthetic DNA

<400> SEQUENCE: 48

tgttgagaaa tggcggcgt 19

<210> SEQ ID NO 49
<211> LENGTH: 31
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 49

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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 50

ucccacugau ucugaauucu uucaa 25

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cuucauccca cugauucuga auucu 25

<210> SEQ ID NO 52
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uuguacuuca ucccacugau ucuga 25

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<212> TYPE: RNA
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<400> SEQUENCE: 53

uguucuugua cuucauccca cugau 25

<210> SEQ ID NO 54
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 54

gaagguguuc uuguacuuca ucccа 25

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<210> SEQ ID NO 55
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<212> TYPE: RNA
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<400> SEQUENCE: 55

guucugaagg uguucuugua cuuca 25

<210> SEQ ID NO 56
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cuccgguucu gaagguguuc uugua 25

<210> SEQ ID NO 57
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<212> TYPE: RNA
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<400> SEQUENCE: 57

guugccuccg guucugaagg uguuc 25

<210> SEQ ID NO 58
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<400> SEQUENCE: 58

caacuguugc cuccgguucu gaagg 25

<210> SEQ ID NO 59
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ucauucaacu guugccuccg guucu 25

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acauuucauu caacuguugc cuccg 25

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<400> SEQUENCE: 61

cuuuaacauu ucauucaacu guugc 25

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gaauccuuua acauuucauu caacu 25

<210> SEQ ID NO 63
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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 63

guguugaauc cuuuaacauu ucauu 25

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<212> TYPE: RNA
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ccauuguguu gaauccuuua acauu 25

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<400> SEQUENCE: 65

uccagccauu guguugaauc cuuua 25

<210> SEQ ID NO 66
<211> LENGTH: 25
<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 66

uagcuuccag ccauuguguu gaauc 25

<210> SEQ ID NO 67
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uuccuuagcu uccagccauu guguu 25

<210> SEQ ID NO 68

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<211> LENGTH: 25
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gcuucuuccu uagcuuccag ccauu 25

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<400> SEQUENCE: 69

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<212> TYPE: RNA
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<220> FEATURE:
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<400> SEQUENCE: 70

gaccugcuca gcuucuuccu uagcu 25

<210> SEQ ID NO 71
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 71

ccuaagaccu gcucagcuuc uuccu 25

<210> SEQ ID NO 72
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 72

ccuguccuaa gaccugcuca gcuuc 25

<210> SEQ ID NO 73
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 73

ucuggccugu ccuaagaccu gcuca 25

<210> SEQ ID NO 74
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 74

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uuggcucugg ccuguccuaa gaccu 25

<210> SEQ ID NO 75
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
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<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 75

caagcuuggc ucuggccugu ccuaa 25

<210> SEQ ID NO 76
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 76

ugacucaagc uuggcucugg ccugu 25

<210> SEQ ID NO 77
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uuccaugacu caagcuuggc ucugg 25

<210> SEQ ID NO 78
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<400> SEQUENCE: 78

ccuccuucca ugacucaagc uuggc 25

<210> SEQ ID NO 79
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<400> SEQUENCE: 79

gggacccucc uuccaugacu caagc 25

<210> SEQ ID NO 80
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
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guauagggac ccuccuucca ugacu 25

<210> SEQ ID NO 81
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<400> SEQUENCE: 81

cuacuguaua gggacccucc uucca 25

<210> SEQ ID NO 82
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 82

ugcaucuacu guauagggac ccucc 25

<210> SEQ ID NO 83
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<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 83

uggauugcau cuacuguaua gggac 25

<210> SEQ ID NO 84
<211> LENGTH: 25
<212> TYPE: RNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic Nucleic Acid

<400> SEQUENCE: 84

ucuuuuggau ugcaucuacu guaua 25

<210> SEQ ID NO 85
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The invention claimed is:

1. An antisense oligomer which causes skipping of the 53rd exon in the human dystrophin gene, consisting of the nucleotide sequence of SEQ ID NO: 57, wherein the antisense oligomer is an oligonucleotide in which the sugar moiety and/or the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is modified, or a morpholino oligomer.

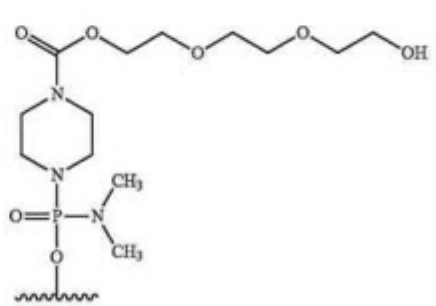
2. The antisense oligomer according to claim 1, wherein the sugar moiety of at least one nucleotide constituting the oligonucleotide is a ribose in which the 2'-OH group is replaced by any one selected from the group consisting of: OR, R, R'OR, SH, SR, NH_2, NHR, NR_2, N_3, CN, F, Cl, Br, and I, wherein R is an alkyl or an aryl and R' is an alkylene.

3. The antisense oligomer according to claim 1, wherein the phosphate-binding region of at least one nucleotide constituting the oligonucleotide is any one selected from the group consisting of: a phosphorothioate bond, a phosphorodithioate bond, an alkylphosphonate bond, a phosphoramidate bond, and a boranophosphate bond.

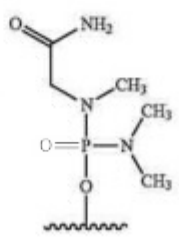
4. The antisense oligomer according to claim 1, which is a morpholino oligomer.

5. The antisense oligomer according to claim 4, which is a phosphorodiamidate morpholino oligomer.

6. The antisense oligomer according to claim 4, wherein the 5' end is any one of the groups of chemical formulae (1) to (3) below:



(1)



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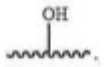
(3)



7. A pharmaceutical composition for the treatment of muscular dystrophy, comprising as an active ingredient the antisense oligomer according to claim 1, or a pharmaceutically acceptable salt or hydrate thereof.

* * * * *

U.S. DEPARTMENT OF COMMERCE
PATENT & TRADEMARK OFFICE

EXHIBIT 30

 Human Molecular Genetics, 2001, Vol. 10, No. 15 **1547–1554**

Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells

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Due to frame-shifting mutations in the DMD gene that cause dystrophin deficiency, Duchenne muscular dystrophy (DMD) patients suffer from lethal muscle degeneration. In contrast, mutations in the allelic Becker muscular dystrophy (BMD) do not disrupt the translational reading frame, resulting in a less severe phenotype. In this study, we explored a genetic therapy aimed at restoring the reading frame in muscle cells from DMD patients through targeted modulation of dystrophin pre-mRNA splicing. Considering that exon 45 is the single most frequently deleted exon in DMD, whereas exon (45+46) deletions cause only a mild form of BMD, we set up an antisense-based system to induce exon 46 skipping from the transcript in cultured myotubes of both mouse and human origin. In myotube cultures from two unrelated DMD patients carrying an exon 45 deletion, the induced skipping of exon 46 in only ~15% of the mRNA led to normal amounts of properly localized dystrophin in at least 75% of myotubes. Our results provide first evidence of highly effective restoration of dystrophin expression from the endogenous gene in DMD patient-derived muscle cells. This strategy may be applicable to not only >65% of DMD mutations, but also many other genetic diseases.

INTRODUCTION

The Duchenne and Becker muscular dystrophies (DMD/BMD) are X-linked muscle diseases typically caused by frame-shifting (DMD) or non-frame-shifting (BMD) mutations in the dystrophin-coding DMD gene (1–4). Dystrophin is an essential component of the dystrophin–glycoprotein complex (DGC) maintaining the membrane integrity of muscle fibers (5–8). In DMD, the absence of dystrophin induces a continuous loss of muscle fibers which eventually causes premature death in adolescence, while in BMD a partly to largely functional dystrophin results in a milder phenotype and a longer life expectancy (2,4).

Given the severity and frequency (1:3500 newborn males) of DMD, great effort has been put into the development of an effective (gene) therapy. Most DMD gene therapy strategies have been based on 'gene addition' through viral or non-viral delivery of dystrophin-encoding sequences to muscle tissue. Studies in mice have been encouraging in showing that expression of dystrophin gene constructs at 20–30% of normal levels is already sufficient to avert muscle degeneration (9). However, two major challenges have hampered clinical applications, the large size of the dystrophin-coding region (11 kb) and the efficient delivery and long-term expression of therapeutic gene constructs into skeletal muscle (reviewed in 10). Alternative DMD gene therapy approaches aiming at gene correction instead of gene addition are therefore gaining increased attention.

Oligonucleotide technology has evolved rapidly in the last decade, facilitating different applications in gene therapy studies. Promising results have been obtained with chimeric DNA/RNA oligonucleotides that are capable of inducing site-specific correction of small DNA mutations using the cellular DNA mismatch repair system (11–13). Such gene correction has been demonstrated in several *in vitro* and *in vivo* disease models, including the mouse (*mdx*) and canine (GRMD) models for DMD (14,15). Although this type of gene correction may be cumulative and potentially even permanent, its efficiency has been limited and depends crucially on DNA repair activity in the host cells of interest.

Antisense oligo(ribo)nucleotides (AONs) have been investigated to modulate gene expression through interference with RNA processing. AONs can block undesired gene expression through RNase H cleavage of RNA–AON duplexes, e.g. for the treatment of viral infections, cancers and inflammatory disorders (16–20), or modulate pre-mRNA splicing by inducing the skipping of specific exons, so that frame-shifting mutations are bypassed and protein synthesis is restored (21). That the latter application may have promising therapeutic potential for DMD has recently been demonstrated in muscle cells from the *mdx* mouse carrying a nonsense point mutation in dystrophin exon 23. Following transfection of AONs directed to the 3′ or 5′ splice sites of exon 23, the skipping of this exon from the transcript was induced, which corrected the translational reading frame and restored dystrophin expression at the sarcolemma both *in vitro* and *in vivo* (22–24). However, these studies have also indicated that antisense targeting of

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3′ or 5′ splice site sequences can cause less predictable skipping of additional adjacent exons. Therefore, in this study we aimed at more specific, internal exon sequences that are involved in the splicing of the upstream intron, such as the polypurine-rich exon recognition sequences (ERSs) or exonic splicing enhancers (ESEs) (25,26). Based on the presence of purine-rich sequences and suboptimal splice sites, several dystrophin exons (e.g. exon 46) may contain such splicing regulatory elements that are potential targets for AONs to induce exon skipping (27,28).

In patients carrying an exon 45 deletion, the single most frequently deleted exon causing DMD, exon 46 skipping would correct the reading frame and generate the shorter dystrophin protein found in the milder affected BMD patients with a deletion of both exons 45 and 46 (Fig. 1A). Here we report highly effective skipping of exon 46 from the transcript using AONs complementary to a putative ERS within this exon. This targeted conversion of a DMD into a BMD mutation induced the synthesis of normal amounts of properly localized dystrophin in cultured muscle cells from two unrelated exon 45 deletion DMD patients.

RESULTS

Exon 46 skipping in mouse muscle cells

We first tested the feasibility of exon 46 skipping in mouse muscle cells. A series of partly overlapping AONs was designed, directed to a polypurine-rich sequence (stretching from cDNA position 6883 to 6953) resembling an ERS-like splicing regulatory element (Fig. 1B). The efficacy of AONs is largely determined by their binding affinity for the target sequence. Due to base composition and pre-mRNA secondary or tertiary structure, it is difficult to predict which AONs are capable of binding the target sequence. Therefore, gel mobility shift assays were performed by hybridization of a ^{32}P-labeled exon 46 RNA fragment to the different deoxy-AONs (mAONs). Five out of the 12 mAONs designed (mAON 4, 6, 8, 9 and 11) (Fig. 1B and Table 1) induced a mobility shift (Fig. 2A), indicating their specific affinity for the target RNA. The binding was both time- and dose-dependent (data not shown). These five mAONs were then analyzed for their efficacy in inducing skipping of dystrophin exon 46 in cultured mouse myotubes. For that purpose, we applied 5′-fluorescein (FAM)-labeled 2′-*O*-methyl phosphorothioate modified oligoribonucleotide-AONs (2′OMe-PS-mAONs), which are resistant to endonucleases and RNaseH and bind to RNA with high affinity (29,30). Upon transfection into mouse myotubes using the cationic polymer polyethylenimine (PEI), typically 60–70% of cells showed specific nuclear uptake of the fluorescent 2′OMe-PS-mAONs (Fig. 2B). At 24 h post-transfection, RT–PCR analysis of myotubes treated with mAON 4, 6, 9 and 11 showed the presence of a novel shorter transcript fragment (324 bp), corresponding in size to exon 45 spliced to exon 47 (Fig. 2C). Sequence analysis of these shorter fragments confirmed the precise skipping of exon 46 (Fig. 2D). Despite its apparent binding affinity in gel mobility shift assays, mAON 8 did not reproducibly induce exon 46 skipping in myotubes. This may have been due to variable binding kinetics. No exon skipping was induced in myotubes following transfection with the non-complementary AON 19, demonstrating

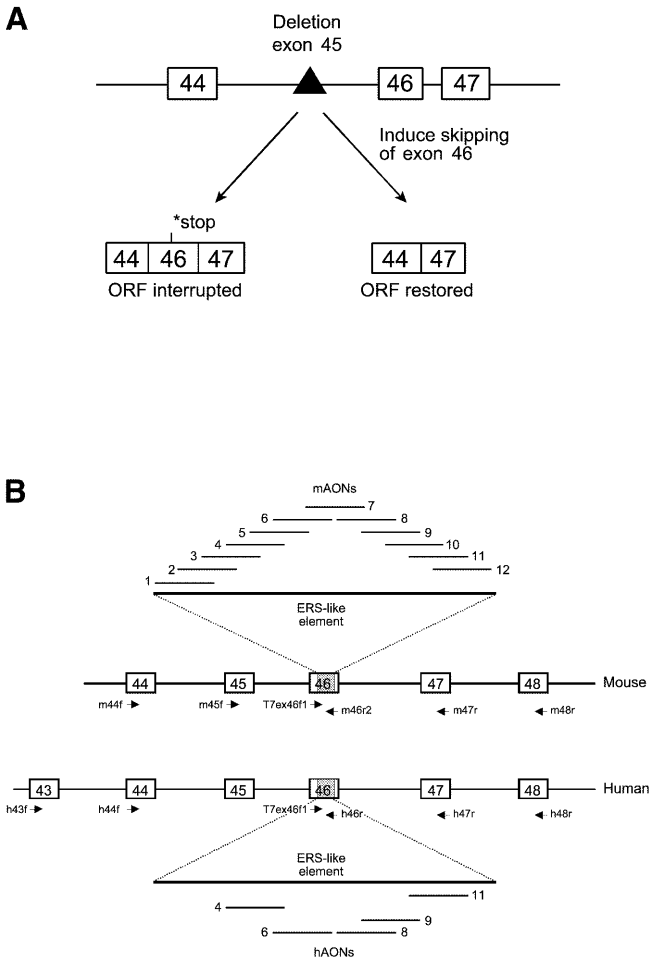

Figure 1. Schematic illustrations of the exon 46 skipping strategy. (**A**) In DMD patients affected by an exon 45 deletion, a stop codon occurs in exon 46, aborting dystrophin synthesis. Induced skipping of exon 46 would restore the open reading frame (ORF) and lead to a shorter, BMD-like protein. (**B**) Binding sites for the various mouse- and human-specific RT–PCR primers and AONs (Table 1). The putative splicing regulatory sequence, resembling an ERS and located between cDNA positions 6883 and 6953, is indicated by the gray box drawn in exon 46.

the sequence-specificity of the AON-mediated exon skipping. The skipping efficiencies of the different mAONs were estimated by visual comparison to a concentration series of control DNA (not shown), and found to range between 5 and 15% of total RT–PCR products.

Exon 46 skipping in human muscle cells

Following the initial set up in mouse cells, we focused on inducing the skipping of exon 46 from the human dystrophin transcript. Primary muscle cells were isolated from muscle biopsies from one healthy individual and two unrelated DMD patients with an exon 45 deletion (DL279.1 and DL272.2). Myotube cultures were prepared and, prior to AON-treatments, tested for their myogenic quality by immunohistochemical analyses with muscle-specific antibodies raised against desmin (stains all muscle cells) and myosin (stains only differentiated myotubes). The differentiating myogenic cell content was determined to be ~25% for the control sample, 25% for DL279.1 and 10% for DL272.2 (Fig. 3A). Staining with two

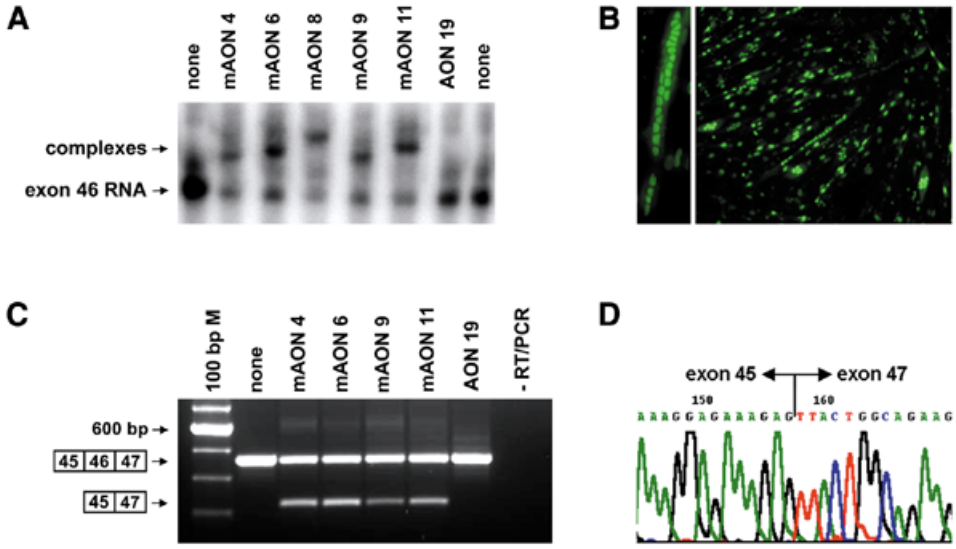

Figure 2. Exon 46 skipping in cultured mouse C2C12 muscle cells. (**A**) Gel mobility shift assay demonstrating the binding of mAON 4, 6, 8, 9 and 11 to the ^{32}P-labeled target exon 46 RNA. Complexes of RNA and AON exhibited a lower gel mobility when compared to free RNA (none). AON 19, a non-complementary control oligoribonucleotide derived from sequences elsewhere in the DMD gene, did not bind to exon 46. (**B**) 5′-Fluorescein-labeled 2′OMe-PS-mAONs were transfected into mouse myotubes. By 24 h post-transfection, 60–70% of myotubes showed multiple fluorescent nuclei indicating successful delivery of the mAONs. The left panel shows an enlargement of one myotube containing more than 20 mAON-positive nuclei. (**C**) RT–PCR analysis of dystrophin mRNA around exon 46. A shorter transcript fragment of 324 bp, corresponding to exon 45 spliced to exon 47, appeared upon treatment with mAON 4, 6, 9 and 11. AON 19 is a non-complementary control. M, 100 bp size marker; –RT/PCR, negative control. (**D**) Sequence analysis of the shorter RT–PCR products confirmed the precise skipping of exon 46.

different monoclonal antibodies against epitopes corresponding to dystrophin exons 31–32 (MANDYS1) and exon 77–79 (Dys2), confirmed the presence of dystrophin at the membranes of the control myotubes, whereas the patient-derived myotubes were dystrophin-negative (Fig. 3A). Five human-specific AONs were designed (hAON 4, 6, 8, 9 and 11) (Fig. 1B and Table 1), corresponding to the mouse-specific AONs that induced the skipping of exon 46 in mouse muscle cells. Gel mobility shift assays with these hAONs showed high affinity of hAON 4, 6, 8 and 9 to labeled human exon 46 RNA (data not shown). Control and patient-derived myotube cultures were then transfected with these hAONs (2′OMe-PS-hAON 4, 6, 8 and 9). Based on the nuclear fluorescence observed, the transfection efficiencies were at least 50%. At 24 h post-transfection, RNA was isolated from the myotubes and analyzed by RT–PCR. Upon treatment with hAON 4, 6 and 8, the control showed a shorter RT–PCR product of 471 bp, corresponding to exons 44 and 45 being directly spliced to exon 47. In the DMD patients, a novel shorter transcript fragment of 295 bp, suggesting a direct splicing of exon 44 to exon 47, appeared in response to the same hAONs (Fig. 3B). The skipping efficiency was reproducibly highest with hAON 8, and estimated (by visual comparison to a concentration series of control DNA) to be up to 15% of total RT–PCR products. Sequence analysis of the RT–PCR products revealed that the shorter fragments were products of the exact skipping of exon 46 from the human dystrophin transcript (Fig. 3C). The AON treatments did not induce any other aberrant dystrophin splicing patterns (data not shown), indicating the high specificity of the strategy.

To assess whether skipping of exon 46 restored the reading frame and dystrophin synthesis in DL279.1 and DL272.2, hAON8-treated myotubes from both patients were fixed at different time points post-transfection, and analyzed with dystrophin antibodies MANDYS1 and Dys2. By 24 h post-transfection, strong dystrophin expression was observed at a level not distinguishable from control myotubes, but still located mostly in the cytoplasm (Fig. 3D). By 48 h, the dystrophin signal had markedly moved to the myotube membrane (Fig. 3D). The levels of dystrophin were still high at day 3, but reduced at days 4 and 5 (data not shown). To determine the percentage of myotubes in which dystrophin expression was restored, the transfected cultures were double-stained with antibodies against myosin (identifies sufficiently differentiated myotubes) and dystrophin. For DL279.1, 42 out of 57 myosin-positive myotubes analyzed (74%) showed dystrophin expression with MANDYS1, and 37 out of 45 (82%) with Dys2. Similar percentages were obtained for DL272.2.

DISCUSSION

Alternative splicing has been observed throughout the DMD gene in numerous BMD/DMD patients, modifying the translational reading frame and adjusting the clinical phenotype (28,31,32). Furthermore, in muscle from DMD patients dystrophin-positive (revertant) fibers can be found, originating from secondary somatic mutations and/or alternative splicing events that restore the translational reading frame (33–35). These findings suggest the potential utility of gene therapy strategies that are based on the targeted manipulation of dystrophin pre-mRNA splicing to restore the reading frame and thus the dystrophin production in muscle cells from DMD patients.

One of the most frequent mutations causing DMD is a deletion of exon 45. The much milder phenotype of BMD patients

Table 1. Sequences of AONs and PCR primers

Name	Sequence (5′→3′)	Remarks
mAON 4	FAM-cugcuucuuccagcc	2′OMe-PS-RNA
mAON 6	FAM-guuaucugcuucuuccagcc	2′OMe-PS-RNA
mAON 8	FAM-guuguucuuuuagcugcugc	2′OMe-PS-RNA
mAON 9	FAM-uuagcugcugcucau	2′OMe-PS-RNA
mAON 11	FAM-cugcugcucaucucc	2′OMe-PS-RNA
AON 19	FAM-ucugcuggcaucuugc	Mouse/human AON directed at dystrophin exon 19
T7ex46f1	*GGATCCTAATACGACTCACTATAGGAACAGACCACCATG*GTCTTGTCAGAATTTCAAAGAG[a]	Mouse/human exon 46 forward primer including 5′ T7 promoter sequence
m46r2	ACTTGTTCAAGTTGTTCTTTTAG	Mouse exon 46 reverse primer
m44f	GCGATTCGACAGATCAGTTG	Mouse exon 44 forward primer
m45f	GAACTCCAGGATGGCATTGG	Mouse exon 45 forward primer
m47r	TTATCCACTGGAGATTTGTCTG	Mouse exon 47 reverse primer
m48r	CTTTATGTCAAACGGTCCTGC	Mouse exon 48 reverse primer
hAON 4	FAM-cugcuuccuccaacc	2′OMe-PS-RNA
hAON 6	FAM-guuaucugcuuccuccaacc	2′OMe-PS-RNA
hAON 8	FAM-gcuuuucuuuuaguugcugc	2′OMe-PS-RNA
hAON 9	FAM-uuaguugcugcucuu	2′OMe-PS-RNA
hAON 11	FAM-uugcugcucuuuucc	2′OMe-PS-RNA
h46r	ACTTGCTCAAGCTTTTCTTTTAG	Human exon 46 reverse primer
h43f	TCTCTCCCAGCTTGATTTCC	Human exon 43 forward primer
h44f	GCGATTTGACAGATCTGTTG	Human exon 44 forward primer
h47r	TTATCCACTGGAGATTTGTCTG	Human exon 47 reverse primer
h48r	CTGAACGTCAAATGGTCCTTC	Human exon 48 reverse primer

[a]The T7 promoter sequence is shown in italic typeface.

carrying an exon 45–46 deletion implies that a dystrophin truncated in this way (lacking amino acids 2147–2254) is still largely functional. We therefore hypothesize that DMD patients with a deletion of exon 45 may benefit significantly from exon 46 skipping therapy. In this study, we show that AONs directed to a putative splicing regulatory sequence in exon 46 induced substantial, specific skipping of this exon from the dystrophin transcript in muscle cells of both mouse and human origin. Exon 46 skipping in muscle cells from patients with an exon 45 deletion restored dystrophin synthesis to control levels and with a correct localization in at least 75% of myotubes. By 24 h post-transfection, the dystrophin was still predominantly located in the cytoplasm. This was to be expected as complete transcription of the 2.4 Mb DMD gene requires 16 h and most splicing occurs co-transcriptionally (36). The expression at 24 h therefore resulted from early translation of matured in-frame transcripts. The dystrophin signals that were detected at the myotube membranes after 48 h indicate that the novel protein was properly routed and accumulated, suggesting at least partial functionality. The dystrophin expression at the membrane was sustained for 3 days post-transfection, followed by a gradual loss after 4 and 5 days. While this may suggest a different half life or stability of the BMD-type dystrophin, it may be at least partly due to the experimental setting. At this phase, myotubes lose viability and are lost from the culture. Moreover, after a few days the concentration of the AONs is expected to be significantly reduced due to limited stability.

Our results demonstrate that AON-based DMD gene therapy can be specific and highly efficient, two important factors to be addressed prior to future clinical applications. Upon AON-treatment of the mouse and human muscle cells, the full-length dystrophin cDNA was checked by RT–PCR analysis using 10 different primer sets (data not shown). Unlike earlier studies in *mdx* mice showing the simultaneous skipping of other exons in addition to exon 23 in muscle cells transfected with different AONs directed to the 3′or 5′ splice sites of exon 23 (22,24), we did not observe any other aberrant splicing patterns. Although non-consecutive exon splicing may cause co-skipping of additional exons in response to AONs, we believe that by directing the AONs not to splice site consensus sequences but to internal exon sequences such as ERSs, a higher level of skipping specificity will be obtained. In addition, a BLAST search of the human EST-database for sequences homologous to the AONs applied in our experiments did not reveal any perfect hits, suggesting a low probability of unintentional exon-skipping through non-specific interactions. The binding of the AONs to exon 46 induced the specific skipping of exon 46 from the

Table 2. Overview of potential target exons for AON-treatment

Exon to skip	Therapeutic for DMD deletions (exons)	Frequency in DMD–Leiden Database (%)
2	3–7	2
8	3–7, 4–7, 5–7, 6–7	4
43	44, 44–47	5
44	35–43, 45, 45–54	8
45	18–44, 44, 46–47, 46–48, 46–49, 46–51, 46–53	13
46	45	7
50	51, 51–55	5
51	50, 45–50, 48–50, 49–50, 52, 52–63	15
52	51, 53, 53–55	3
53	45–52, 48–52, 49–52, 50–52, 52	9

Skipping of these exons would restore the reading frame in series of patients affected by different deletions of one or more exons. Their total frequency in the DMD–Leiden Database (http://www.dmd.nl) is indicated.

dystrophin transcripts. Although we may not exclude the possibility that the binding itself disturbs the RNA secondary structure which inhibits exon 46 splicing, our data strongly suggests that the sequence targeted in these studies indeed acts as a splicing regulatory, ERS-like, element.

The efficiency of the exon 46 skipping therapy was determined through immunohistochemical analyses. In transfected myotube cultures derived from two unrelated DMD patients, the percentages of myosin-positive myotubes that also expressed dystrophin were 74 and 82%. These are remarkably high efficiencies since the AON transfection efficiencies were qualitatively estimated to be ~50%, and exon 46 skipping only occurred in ~15% of DMD transcripts (of all nuclei). Apparently, not all nuclei within one myotube need to be transfected, and a relatively low amount of corrected in-frame transcripts in the transfected nuclei is sufficient to accumulate almost normal quantities of dystrophin throughout the muscle fiber. This is consistent with studies in mice showing that restoration of dystrophin expression to 20–30% of normal levels is sufficient to avert muscle degeneration (9).

Our results provide promising proof of principle for antisense-based therapy for DMD. Due to their synthetic and sequence-specific nature, the application of AONs for therapeutic purposes is relatively safe in comparison with gene therapy based on viral vectors. Moreover, the targeted modulation of dystrophin pre-mRNA splicing offers important advantages, such as the simultaneous correction of most or all affected dystrophin isoforms and, most likely, the maintenance of the original (tissue-)specific gene regulation. This strategy may be applicable to a variety of DMD mutations. As most DMD-causing deletions are clustered in two mutation hot spots, the targeted skipping of one particular exon may restore the reading frame in a series of patients with different mutations. A survey of the Leiden DMD Database (http://www.dmd.nl) reveals that through skipping of only 10 different exons, >65% of DMD deletion mutations may already be convertible into their nearest adjacent BMD counterpart (Table 2). On-going studies aim to target these exons for AON-induced skipping in human muscle cells *in vitro*. In addition, to facilitate *in vivo* applications, we are currently studying different strategies for the efficient delivery of AONs to mouse muscle tissue.

MATERIALS AND METHODS

Oligonucleotides

For the gel mobility shift assays, we used HPLC-purified oligodeoxynucleotides (EuroGentec, Belgium). The AONs with highest binding affinity for the target RNA were re-synthesized to contain a FAM group, a phosphorothioate modified backbone and 2′OMe-PS-RNA molecules, and were HPLC purified (EuroGentec, Belgium). To avoid interference in immunohistochemical analysis, we also used a 2′OMe-PS-RNA-hAON8 without a 5′-fluorescein label. All AONs and RT–PCR primers are depicted in Figure 1B and described in Table 1.

Gel mobility shift assay

Dystrophin exon 46 was amplified from mouse or human genomic DNA using primer combinations T7ex46f1 and m46r2 or T7ex46f1 and h46r (Table 1). The T7ex46f1 primer sequence is identical for mouse and human exon 46, and contains a T7 promoter sequence to facilitate T7 *in vitro* transcription. The PCR fragments (160 bp) were gel purified using the QIAquick Gel Extraction Kit (Qiagen). The *in vitro* transcription was performed using the Riboprobe System-T7 (Promega) according to the manufacturer's instructions, and in the presence of 60 µCi [^{32}P]CTP. The radiolabeled transcript was isolated from a 6% denaturing polyacrylamide gel through overnight incubation in a gel elution buffer (0.5 M ammonium acetate, 2 mM EDTA, 0.2% SDS) at 37°C. The binding affinity of the individual AONs (0.5 pmol) was determined by hybridization at 37°C for 30 min in a hybridization buffer (1 mM Tris–HCl pH 7.4, 50 mM NaCl, 5 mM $MgCl_2$), followed by 8% PAGE and visualization using a PhosphoImager (Molecular Dynamics).

Cell culture and transfection

Mouse myoblast cells derived from the C2C12 cell line (CRL-1772) were obtained from, and grown as recommended by ATCC. Primary human myoblasts were isolated by enzymatic dissociation and cultured as described previously (37). Both mouse and human myoblast cultures were seeded in collagen pre-coated flasks and plates. Myotubes were obtained from confluent myoblast cultures following 10–14 days of serum-deprivation. For transfection of myotubes, we used polyethylenimine (PEI) according to the manufacturer's instructions (ExGen500; MBI Fermentas). The cultures were transfected for 3 h in low-serum medium with 1 µM of each AON linked to PEI at a ratio-equivalent of 3 (mouse myotubes) or 3.5 (human myotubes).

RNA isolation and RT–PCR analysis

At 24 h post-transfection, total RNA was isolated from the myotube cell cultures (RNAzol B; Campro Scientific). An aliquot of 1 µg of RNA was used for RT–PCR analysis using C. therm polymerase (Roche Diagnostics) in a 20 µl reaction at 62°C for 30 min, primed with m48r or h48r. A first amplification was carried out with the outer primers m44f–m48r or h43f–h48r, for 20 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 1 min. A sample of 1 µl of this reaction was then re-amplified using the nested primer combinations m45f–m47r or h44f–h47r for 32 cycles of 94°C for 45 s, 60°C for 45 s and 72°C for 1 min. PCR products were analyzed on 2% agarose gels.

Sequence analysis

RT–PCR products were isolated from 2% agarose gels using the QIAquick Gel Extraction Kit (Qiagen). Direct DNA sequencing was carried out by the Leiden Genome Technology Center (LGTC) using the BigDye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems) and analyzed on an ABI 3700 Sequencer (PE Applied Biosystems).

Immunohistochemical analysis

For immunohistochemical analyses, cells were grown and transfected on cover slips. At different time-points post-transfection (24, 48, 72, 96 or 120 h), cells were fixed in –20°C methanol. Prior to staining with the different antibodies, the cells were incubated for 1 h in a blocking solution containing 5% horse serum (Gibco BRL) and 0.05% Tween-20 (Sigma) in PBS (Gibco BRL). Antibodies were diluted in this blocking solution. The following primary antibodies were used: desmin polyclonal antibody (ICN Biomedicals) diluted 1:100, myosin

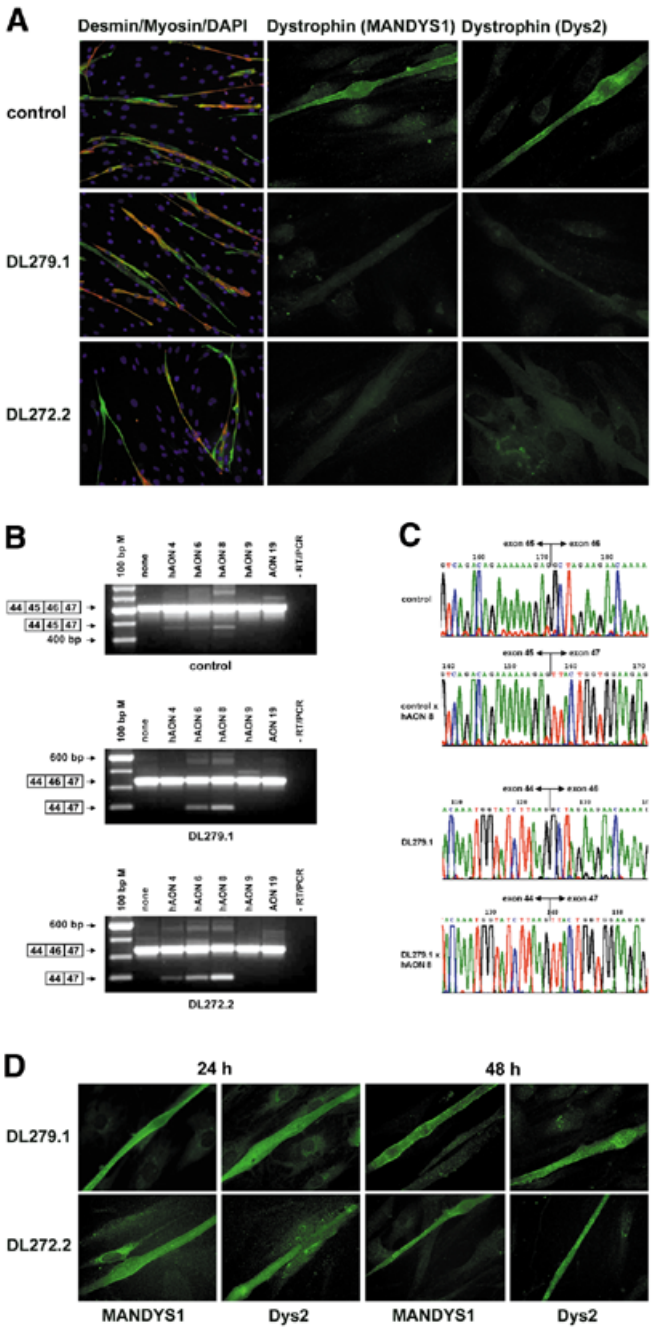

Figure 3. Exon 46 skipping in cultured human primary muscle cells. (**A**) Immunohistochemical analysis of control and DMD-patient (DL279.1 and DL272.2) myotube cultures prior to hAON-treatment. Cells were stained for DAPI (blue) to determine the number of nuclei, desmin (green fluorescence) to identify muscle cells, and myosin (red fluorescence) to differentiate matured myotubes. The myogenic quality (i.e. the percentage of myotubes) of the cultures was 25% for the control sample, 25% for DL279.1 and 10% for DL272.2. Using monoclonal antibodies MANDYS1 (middle panel) and Dys2 (right panel) positive dystrophin signals were detected at the membranes of control myotubes, whereas the patient-derived myotubes were negative for both antibodies. Magnifications: left panel 16×; middle and right panel 63×. (**B**) RT–PCR analysis at 24 h post-transfection. In response to treatment with hAON 4, 6 and 8, novel shorter transcript fragments were detected in the control (471 bp) and in the patients DL279.1 and DL272.2 (295 bp). It is noteworthy that the intensity of the shorter transcript in the control was reproducibly lower than that observed in the patients. This may be explained by preferential degradation of this out-of-frame transcript by nonsense-mediated mRNA decay. AON 19 is the non-complementary control (none, non-transfected myotubes; M, 100 bp size marker; –RT/PCR, negative control). (**C**) Sequence analysis of RT–PCR products indicating that hAON 8 induces the precise skipping of exon 46, generating shorter transcripts containing exon 45 spliced to exon 47 in the control, and exon 44 spliced to exon 47 in DL279.1. (**D**) Immunohistochemical analysis (using MANDYS1 and Dys2) of DL279.1 and DL272.2 myotubes fixed at 24 and 48 h after treatment with hAON 8. In both patients, exon 46 skipping restored dystrophin synthesis. By 24 h, the dystrophin expression was mainly located in the cytoplasm, whereas by 48 h dystrophin signals were also detected at the myotube membranes. Magnification: 63×.

monoclonal antibody (MF20; Developmental Studies Hybridoma Bank, University of Iowa) or polyclonal antibody [L53 (38), a gift from Dr M. van den Hoff, AMC, The Netherlands], both diluted 1:100, and the dystrophin monoclonal antibodies NCL-dys2 (Novocastra Laboratories Ltd) and MANDYS1 (a gift from Dr G. Morris) diluted 1:10. After rinsing, the secondary antibodies Alexa Fluor 594 goat anti-rabbit conjugate (diluted 1:1000) and Alexa Fluor 488 goat anti-mouse conjugate (diluted 1:250) (Molecular Probes Inc.) were applied for 1 h. The coverslips were finally inverted and mounted on glass slides in a Gelvatol solution (39) and analyzed using a Leica confocal microscope equipped with epifluorescence optics. Digital images were captured using a CCD camera (Photometrics).

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EXHIBIT 31

HIGHLIGHTS OF PRESCRIBING INFORMATION
These highlights do not include all the information needed to use VILTEPSO™ safely and effectively. See full prescribing information for VILTEPSO.

VILTEPSO (viltolarsen) injection, for intravenous use
Initial U.S. Approval: 2020

----------RECENT MAJOR CHANGES----------
Dosage and Administratin (2.1), Monitoring to Assess Safety 3/2021
Warnings and Precautions (5.1), Kidney Toxicity 3/2021

----------INDICATIONS AND USAGE----------
VILTEPSO is an antisense oligonucleotide indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the DMD gene that is amenable to exon 53 skipping. This indication is approved under accelerated approval based on an increase in dystrophin production in skeletal muscle observed in patients treated with VILTEPSO. Continued approval for this indication may be contingent upon verification and description of clinical benefit in a confirmatory trial. (1)

----------DOSAGE AND ADMINISTRATION----------
- Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VILTEPSO. (2.1)
- Recommended dosage is 80 milligrams per kilogram of body weight once weekly. (2.2)
- Administer as an intravenous infusion over 60 minutes. (2.2, 2.4)
- If the volume of VILTEPSO required is less than 100 mL, dilution in 0.9% Sodium Chloride Injection, USP, is required. (2.3)

----------DOSAGE FORMS AND STRENGTHS----------
Injection: 250 mg/5 mL (50 mg/mL) in a single-dose vial (3)

----------CONTRAINDICATIONS----------
None (4)

----------WARNINGS AND PRECAUTIONS----------
Kidney Toxicity: Based on animal data, may cause kidney toxicity. Kidney function should be monitored; creatinine may not be a reliable measure of renal function in DMD patients. (5.1, 13.2)

----------ADVERSE REACTIONS----------
The most common adverse reactions (incidence ≥15% in patients treated with VILTEPSO) were upper respiratory tract infection, injection site reaction, cough, and pyrexia. (6.1)

To report SUSPECTED ADVERSE REACTIONS, contact NS Pharma at 1-866 NSPHARM (1-866-677-4276) or FDA at 1-800-FDA-1088 or *www.fda.gov/medwatch*.

See 17 for PATIENT COUNSELING INFORMATION

Revised: 3/2021

FULL PRESCRIBING INFORMATION: CONTENTS*

* Sections or subsections omitted from the full prescribing information are not listed.

FULL PRESCRIBING INFORMATION

1 INDICATIONS AND USAGE

VILTEPSO is indicated for the treatment of Duchenne muscular dystrophy (DMD) in patients who have a confirmed mutation of the DMD gene that is amenable to exon 53 skipping. This indication is approved under accelerated approval based on an increase in dystrophin production in skeletal muscle observed in patients treated with VILTEPSO *[see Clinical Studies (14)].* Continued approval for this indication may be contingent upon verification and description of clinical benefit in a confirmatory trial.

2 DOSAGE AND ADMINISTRATION

2.1 Monitoring to Assess Safety

Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VILTEPSO. Consider measurement of glomerular filtration rate prior to initiation of VILTEPSO. Monitoring for kidney toxicity during treatment is recommended. Obtain the urine samples prior to infusion of VILTEPSO or at least 48 hours after the most recent infusion *[see Warnings and Precautions (5.1)].*

2.2 Dosing Information

The recommended dosage of VILTEPSO is 80 mg/kg administered once weekly as a 60-minute intravenous infusion.

If a dose of VILTEPSO is missed, it should be administered as soon as possible after the scheduled dose time.

2.3 Preparation Instructions

Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration, whenever solution and container permit. Prepare the VILTEPSO dose using aseptic technique.

a. Calculate the total dose of VILTEPSO to be administered based on the patient's weight and the recommended dosage of 80 mg/kg. Determine the volume of VILTEPSO needed and the correct number of vials to supply the full calculated dose.
b. Allow vials to warm to room temperature. Mix the contents of each vial by gently inverting 2 to 3 times. Do not shake.
c. Visually inspect each vial of VILTEPSO. VILTEPSO is a clear and colorless solution. Do not use if the solution in the vials is discolored or particulate matter is present.
d. Withdraw the calculated volume of VILTEPSO from the appropriate number of vials.
    i. If the volume of VILTEPSO required is less than 100 mL, dilution in 0.9% Sodium Chloride Injection, USP is required. Withdraw from the 100-mL infusion bag a volume of 0.9% Sodium Chloride Injection, USP, equivalent to the calculated volume of VILTEPSO and inject the VILTEPSO into the infusion bag, such that the total volume in the bag is 100 mL.

ii. If the volume of VILTEPSO required is 100 mL or more, dilution is not required, and the required amount of VILTEPSO should be placed into an empty infusion bag.

e. Visually inspect the infusion bag containing the solution for particulates. Gently invert the infusion bag to ensure equal distribution of product. Do not shake.
f. VILTEPSO contains no preservatives. Infusion should begin as soon as possible, but no more than 5 hours after preparation of VILTEPSO, and be completed within 6 hours of preparation (allowing for 1 hour of infusion time), if diluted solution is stored at 20°C to 26°C (68°F to 79°F). If immediate use is not possible, the solution may be stored for up to 24 hours at 2°C to 8°C (36°F to 46°F). Do not freeze.
g. VILTEPSO is supplied in single-dose vials. Discard unused VILTEPSO.

2.4 Administration Instructions

VILTEPSO is administered via intravenous infusion using a peripheral or central venous catheter. Flush the intravenous access line with 0.9% Sodium Chloride Injection, USP, after infusion. Filtration of VILTEPSO is not required.

Infuse VILTEPSO over 60 minutes. Do not mix other medications with VILTEPSO or infuse other medications concomitantly via the same intravenous access line. VILTEPSO should be mixed with 0.9% Sodium Chloride Injection, USP, only.

3 DOSAGE FORMS AND STRENGTHS

VILTEPSO is a clear and colorless solution available as follows:

- Injection: 250 mg/5 mL (50 mg/mL) solution in a single-dose vial

4 CONTRAINDICATIONS

None.

5 WARNINGS AND PRECAUTIONS

5.1 Kidney Toxicity

Kidney toxicity was observed in animals who received viltolarsen *[see Use in Specific Populations (8.4)].* Although kidney toxicity was not observed in the clinical studies with VILTEPSO, the clinical experience with VILTEPSO is limited, and kidney toxicity, including potentially fatal glomerulonephritis, has been observed after administration of some antisense oligonucleotides. Kidney function should be monitored in patients taking VILTEPSO. Because of the effect of reduced skeletal muscle mass on creatinine measurements, serum creatinine may not be a reliable measure of kidney function in DMD patients. Serum cystatin C, urine dipstick, and urine protein-to-creatinine ratio should be measured before starting VILTEPSO. Consider also measuring glomerular filtration rate using an exogenous filtration marker before starting VILTEPSO. During treatment, monitor urine dipstick every month, and serum cystatin C and urine protein-to-creatinine ratio every three months. Only urine expected to be free of excreted VILTEPSO should be used for monitoring of urine protein. Urine obtained on the day of VILTEPSO infusion prior to the infusion, or urine obtained at least 48 hours after the most recent infusion, may be used. Alternatively, use a laboratory test that does not use the reagent pyrogallol red, as this reagent has the potential to cross react with any VILTEPSO that is excreted in the urine and thus lead to a false positive result for urine protein.

If a persistent increase in serum cystatin C or proteinuria is detected, refer to a pediatric nephrologist for further evaluation.

6 ADVERSE REACTIONS

6.1 Clinical Trials Experience

Because clinical trials are conducted under widely varying conditions, adverse reaction rates observed in the clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not reflect the rates observed in practice.

In clinical trials with VILTEPSO, 32 patients have been exposed to VILTEPSO once weekly, ranging between 40 mg/kg (0.5 times the recommended dosage) and 80 mg/kg (the recommended dosage), including 16 patients treated for greater than 12 months and 8 patients treated for greater than 24 months as part of an ongoing open-label extension study. All patients were male and had genetically confirmed DMD.

Study 1 was a multicenter, 2-period, dose-finding study conducted in the United States and Canada in males 4 years to less than 10 years of age on a stable corticosteroid regimen for at least 3 months. During the initial period (first 4 weeks) of Study 1, patients were randomized (double-blind) to VILTEPSO or placebo. All patients then received 20 weeks of VILTEPSO 40 mg/kg once weekly (0.5 times the recommended dose) (N=8), or 80 mg/kg once weekly (N=8) *[see Clinical Studies (14)].*

Study 2 was a multicenter, parallel-group, open-label, dose-finding study conducted in Japan. Eligible patients included ambulatory and non-ambulatory males 5 years to less than 18 years of age who were assigned to receive intravenous VILTEPSO 40 mg/kg once weekly (0.5 times the recommended dose) (N=8) or 80 mg/kg once weekly (N=8) for 24 weeks.

Adverse reactions reported in ≥10% of patients treated with VILTEPSO 80 mg/kg/wk in pooled Studies 1 and 2 are displayed in Table 1. The most common adverse reactions (incidence ≥15% in patients treated with VILTEPSO) were upper respiratory tract infection, injection site reaction, cough, and pyrexia. Patients in the pooled analysis were treated with VILTEPSO for 20 to 24 weeks.

Table 1: Adverse Reactions Reported in ≥10% of DMD Patients Treated with VILTEPSO 80 mg/kg Once Weekly (Pooled Studies 1 and 2)

Adverse Reaction	**VILTEPSO 80 mg/kg Once Weekly (n=16) %**
Upper respiratory tract infection*	63
Injection site reaction**	25
Cough	19
Pyrexia	19

Contusion	13
Arthralgia	13
Diarrhea	13
Vomiting	13
Abdominal pain	13
Ejection fraction decreased	13
Urticaria	13

* Upper respiratory tract infection includes the following terms: upper respiratory tract infection, nasopharyngitis, and rhinorrhea.

** Injection site reaction includes the following terms: injection site bruising, injection site erythema, injection site reaction, and injection site swelling.

6.2 Immunogenicity

As with all oligonucleotides, there is potential for immunogenicity. The detection of antibody formation is highly dependent on the sensitivity and specificity of the assay. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including assay methodology, sample handling, timing of sample collection, concomitant medications, and underlying disease. For these reasons, comparison of the incidence of antibodies in the studies described below with the incidence of antibodies in other studies may be misleading.

For Study 1, samples collected from all 16 patients at Day 1 (pre-dose), Week 5, Week 13, and Week 24 were assessed for anti-viltolarsen antibodies. All samples were determined to be antibody negative. For the same study, serum samples collected from all 16 patients at Day 1 (pre-dose), Week 13, and Week 24 were analyzed for anti-dystrophin antibodies. Anti-dystrophin antibodies were detected in 1 out of 16 patients (6.25%) at Weeks 13 and 24; however, at Weeks 37, 49, 73, and 97, no anti-dystrophin antibodies were detected in the same patient. Further, this patient achieved a change from baseline in dystrophin levels that was comparable to the mean change in his dosage group (80 mg/kg/week) and there were no adverse events reported with this antibody production. For Study 2, all samples collected from the 16 patients were determined to be both anti-viltolarsen antibody and anti-dystrophin antibody negative. Overall, there was a lack of observed immunogenicity, which indicates that viltolarsen is not highly immunogenic.

8 USE IN SPECIFIC POPULATIONS

8.1 Pregnancy

Risk Summary

There are no human or animal data available to assess the use of VILTEPSO during pregnancy. In the U.S. general population, major birth defects occur in 2 to 4%, and miscarriage occurs in 15 to 20% of clinically recognized pregnancies.

8.2 Lactation

Risk Summary

There are no human or animal data to assess the effect of VILTEPSO on milk production, the presence of viltolarsen in milk, or the effects of VILTEPSO on the breastfed infant.

The developmental and health benefits of breastfeeding should be considered along with the mother's clinical need for VILTEPSO and any potential adverse effects on the breastfed infant from VILTEPSO or from the underlying maternal condition.

8.4 Pediatric Use

VILTEPSO is indicated for the treatment of DMD in patients who have a confirmed mutation of the DMD gene that is amenable to exon 53 skipping, including pediatric patients *[see Clinical Studies (14)].*

Juvenile Animal Toxicity Data

Viltolarsen (0, 15, 60, 240, or 1200 mg/kg) was administered to juvenile male mice by subcutaneous injection on postnatal day (PND) 7 and by intravenous injection weekly from PND 14 to PND 70. The highest dose resulted in deaths because of renal toxicity. In surviving animals at 240 and 1200 mg/kg, there was a dose-dependent increase in the incidence and severity of renal tubular effects (including degeneration), which were not accompanied by clinical pathology correlates. Reduced body weight gain and delayed sexual maturation were observed at the highest dose tested. At the no-effect dose for renal toxicity (60 mg/kg), plasma exposures were similar to that in humans at the recommended human dose of 80 mg/kg/week.

8.5 Geriatric Use

DMD is largely a disease of children and young adults; therefore, there is no geriatric experience with VILTEPSO.

8.6 Patients with Renal Impairment

VILTEPSO has not been studied in patients with renal impairment. Viltolarsen is mostly excreted unchanged in the urine, and renal impairment may increase its exposure. However, because of the effect of reduced skeletal muscle mass on creatinine measurements in DMD patients, no specific dosage adjustment can be recommended for DMD patients with renal impairment based on estimated glomerular filtration rate. Patients with known renal function impairment should be closely monitored during treatment with VILTEPSO.

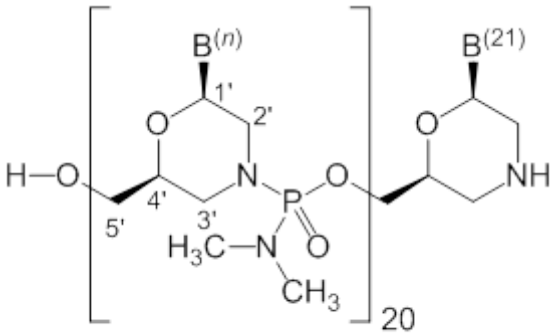
11 DESCRIPTION

VILTEPSO (viltolarsen) injection is a sterile, preservative-free, aqueous solution for intravenous administration. VILTEPSO is a clear and colorless solution. VILTEPSO is supplied in single-dose vials containing 250 mg/5 mL viltolarsen (50 mg/mL) in 0.9% sodium chloride. Each milliliter of VILTEPSO contains 50 mg viltolarsen and 9 mg sodium chloride in water for injection. The final product is adjusted to a pH ranging between 7.0 and 7.5 using hydrochloric acid and/or sodium hydroxide.

Viltolarsen is an antisense oligonucleotide of the phosphorodiamidate morpholino oligomer (PMO) subclass. PMOs are synthetic molecules in which the five-membered ribofuranosyl rings found in natural DNA and RNA are replaced by a six-membered morpholino ring. Each morpholino ring is

linked through an uncharged phosphorodiamidate moiety rather than the negatively charged phosphate linkage that is present in natural DNA and RNA. Each phosphorodiamidate morpholino subunit contains one of the heterocyclic bases found in DNA (adenine, cytosine, guanine, or thymine). Viltolarsen contains 21 linked subunits. The molecular formula of viltolarsen is $C_{244}H_{381}N_{113}O_{88}P_{20}$ and the molecular weight is 6924.82 daltons. The structure and base sequence of viltolarsen are shown in Figure 1.

Figure 1: Structural Formula of Viltolarsen



CCTCCGGTTC TGAAGGTGTT C

12 CLINICAL PHARMACOLOGY

12.1 Mechanism of Action

VILTEPSO is designed to bind to exon 53 of dystrophin pre-mRNA resulting in exclusion of this exon during mRNA processing in patients with genetic mutations that are amenable to exon 53 skipping. Exon 53 skipping is intended to allow for production of an internally truncated dystrophin protein in patients with genetic mutations that are amenable to exon 53 skipping.

12.2 Pharmacodynamics

After treatment with VILTEPSO 80 mg/kg once weekly, all patients evaluated (N=8) were found to produce mRNA for a truncated dystrophin protein, as measured by reverse transcription polymerase chain reaction (RT-PCR), and demonstrated exon 53 skipping, as measured by DNA sequence analysis.

In Study 1, all patients who received VILTEPSO 80 mg/kg once weekly for 20 to 24 weeks showed an increase from baseline in dystrophin protein expression, as quantified by a validated Western blot method (mean 5.3%; median 3.8%; range 0.7% to 13.9% of normal levels when normalized to myosin heavy chain; p-value 0.01). Mass spectrometry, immunofluorescence staining, and RT-PCR results were supportive of the Western blot data *[see Clinical Studies (14)].* Expected localization of truncated dystrophin to the sarcolemma in muscle fibers of patients treated with viltolarsen was confirmed by immunofluorescence staining.

12.3 Pharmacokinetics

The pharmacokinetics of viltolarsen was evaluated in DMD patients following administration of intravenous (IV) doses ranging from 1.25 mg/kg/week (0.016 times the recommended dosage) to 80

mg/kg/week (the recommended dosage). Viltolarsen exposure increased proportionally with dose, with minimal accumulation with once-weekly dosing. Inter-subject variability (as %CV) for C_{max} and AUC ranged from 16% to 27% respectively.

VILTEPSO is administered as an IV infusion over 60 minutes. Bioavailability is assumed to be 100%, and median T_{max} was around 1 hour (end of infusion).

Distribution

The mean viltolarsen steady-state volume of distribution was 300 mL/kg (%CV=14 at a dose of 80 mg/kg. Viltolarsen plasma protein binding ranged from 39% to 40% and is not concentration dependent.

Elimination

Metabolism

Data from in vitro metabolism indicate that viltolarsen is metabolically stable. No metabolites were detected in plasma or urine.

Excretion

VILTEPSO is excreted mainly as an unchanged drug in the urine. Viltolarsen elimination half-life was 2.5 (%CV=8) hours, and plasma clearance was 217 mL/hr/kg (%CV=22).

Specific Populations

Age, Sex & Race

The pharmacokinetics of viltolarsen have been evaluated only in male pediatric DMD patients.There is no experience with VILTEPSO in patients 65 years of age or older. No marked differences in any PK parameters were observed between White and Asian patients.

Patients with Renal or Hepatic Impairment

VILTEPSO has not been studied in patients with renal or hepatic impairment. Viltolarsen was found to be metabolically stable, and hepatic metabolism does not contribute to the elimination of viltolarsen. In addition, viltolarsen was mainly excreted unchanged in the urine. Viltolarsen is eliminated renally, and renal impairment is expected to result in increasing exposure of viltolarsen. However, because of the effect of reduced skeletal muscle mass on creatinine measurements in DMD patients, no specific dosage adjustment can be recommended for DMD patients with renal impairment based on glomerular filtration rate estimated by serum creatinine *[see Use in Specific Populations (8.6)].*

In Vitro Drug Interaction Studies

Viltolarsen did not inhibit CYP3A4/5, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, UGT1A1, or UGT2B7. Viltolarsen did not induce CYP1A2, CYP2B6, or CYP3A4.

Viltolarsen is not metabolized by CYP enzymes and is not a substrate of transporters BCRP, BSEP, MDR1, OAT1, OAT3, OCT1, OCT2, MATE1, or MATE2-K. Viltolarsen did not inhibit the transporters tested (OATP1B1, OATP1B3, OAT3, BCRP, MDR1, BSEP, OAT1, OCT1, OCT2, MATE1, and MATE2-K).

Based on in vitro data, viltolarsen has a low potential for drug-drug interactions with major CYP enzymes and drug transporters in humans.

13 NONCLINICAL TOXICOLOGY

13.1 Carcinogenesis, Mutagenesis, Impairment of Fertility

Carcinogenesis

Carcinogenicity studies of viltolarsen have not been conducted.

Mutagenesis

Viltolarsen was negative for genotoxicity in *in vitro* (bacterial reverse mutation, chromosomal aberration in Chinese hamster lung cells) and *in vivo* (mouse bone marrow micronucleus) assays.

Impairment of Fertility

Intravenous administration of viltolarsen (0, 60, 240, or 1000 mg/kg) to male mice weekly prior to and during mating to untreated females did not have adverse effects on fertility. Plasma exposure (AUC) at the highest dose was approximately 18 times that in humans at the recommended human dose of 80 mg/kg/week.

14 CLINICAL STUDIES

The effect of VILTEPSO on dystrophin production was evaluated in one study in DMD patients with a confirmed mutation of the DMD gene that is amenable to exon 53 skipping (Study 1; NCT02740972).

Study 1 was a multicenter, 2-period, dose-finding study conducted in the United States and Canada. During the initial period (first 4 weeks) of Study 1, patients were randomized (double blind) to VILTEPSO or placebo. All patients then received 20 weeks of open-label VILTEPSO 40 mg/kg once weekly (0.5 times the recommended dosage) (N=8) or 80 mg/kg once weekly (N=8). Study 1 enrolled ambulatory male patients 4 years to less than 10 years of age (median age 7 years) on a stable corticosteroid regimen for at least 3 months.

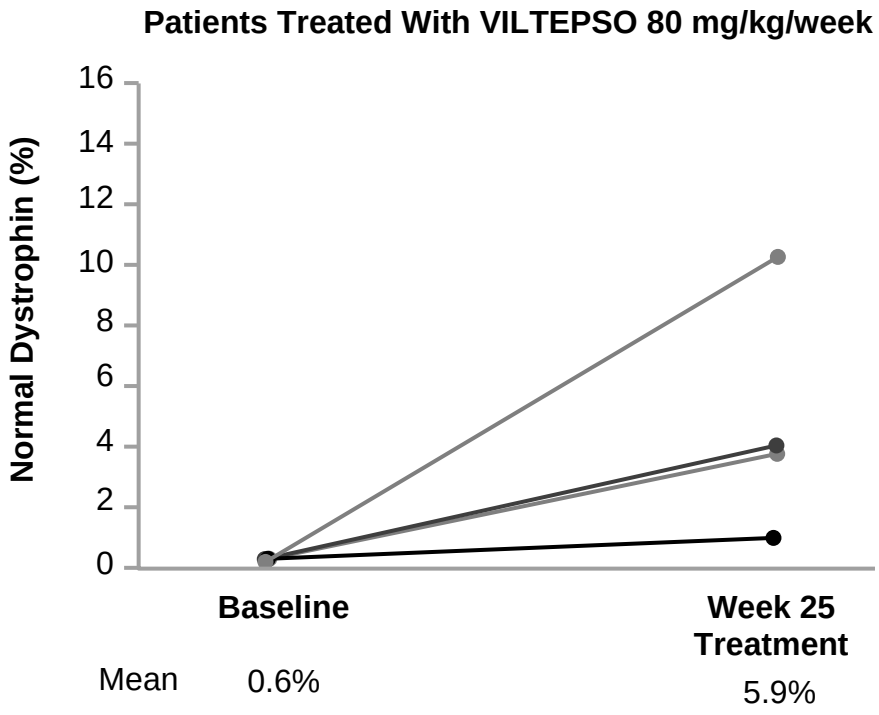
Efficacy was assessed based on change from baseline in dystrophin protein level (measured as % of the dystrophin level in healthy subjects, i.e., % of normal) at Week 25. Muscle biopsies (left or right biceps brachii) were collected from patients at baseline and following 24 weeks of VILTEPSO treatment, and analyzed for dystrophin protein level by Western blot normalized to myosin heavy chain (primary endpoint) and mass spectrometry (secondary endpoint).

In patients who received VILTEPSO 80 mg/kg once weekly, mean dystrophin levels increased from 0.6% (SD 0.8) of normal at baseline to 5.9% (SD 4.5) of normal by Week 25, with a mean change in dystrophin of 5.3% (SD 4.5) of normal levels (p=0.01) as assessed by validated Western blot

(normalized to myosin heavy chain); the median change from baseline was 3.8%. All patients demonstrated an increase in dystrophin levels over their baseline values. As assessed by mass spectrometry (normalized to filamin C), mean dystrophin levels increased from 0.6% (SD 0.2) of normal at baseline to 4.2% (SD 3.7) of normal by Week 25, with a mean change in dystrophin of 3.7% (SD 3.8) of normal levels (nominal p=0.03, not adjusted for multiple comparisons); the median change from baseline was 1.9%.

Individual patient dystrophin levels in patients evaluated in Study 1 are shown in Figure 2 and Table 2.

Figure 2: Dystrophin Expression in Individual Patients (Study 1)



Note: Solid lines represent individual patient data. Dystrophin was measured using Western blot and normalized to myosin heavy chain.

Table 2: Dystrophin Expression in Individual Patients (Study 1)

Patient Number	Western Blot % Normal Dystrophin[a]		
	Baseline	Week 25	Change from Baseline
1	0.46	1.14	0.69
2	0.40	3.97	3.57
3	0.46	2.97	2.51
4	0.09	10.40	10.31
5	0.51	14.42	13.91
6	2.61	7.40	4.79
7	0.43	3.06	2.63
8	0.09	4.07	3.98

[a] Data were normalized by myosin heavy chain

16 HOW SUPPLIED/STORAGE AND HANDLING

16.1 How Supplied

VILTEPSO injection is supplied in single-dose vials. The solution is clear and colorless.

- Single-dose vials containing 250 mg/5 mL (50 mg/mL) viltolarsen NDC 73292-011-01

16.2 Storage and Handling

Store VILTEPSO at 2°C to 8°C (36°F to 46°F). Do not freeze.

17 Patient Counseling Information

Kidney Toxicity

Inform patients nephrotoxicity has occurred with drugs similar to VILTEPSO. Advise patients of the importance of monitoring for kidney toxicity by their healthcare providers during treatment with VILTEPSO *[see Warnings and Precautions (5.1)].*

Manufactured for:
NS Pharma, Inc.
Paramus, NJ 07652

EXHIBIT 32

Opposition to EP 1619249
Document D16

PERGAMON

Neuromuscular Disorders 9 (1999) 330–338

Specific removal of the nonsense mutation from the *mdx* dystrophin mRNA using antisense oligonucleotides

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Abstract

The *mdx* mouse, which carries a nonsense mutation in exon 23 of the dystrophin gene, has been used as an animal model of Duchenne muscular dystrophy to evaluate cell or gene replacement therapies. Despite the *mdx* mutation, which should preclude the synthesis of a functional dystrophin protein, rare, naturally occurring dystrophin-positive fibres have been observed in *mdx* muscle tissue. These dystrophin-positive fibres are thought to have arisen from an exon-skipping mechanism, either somatic mutations or alternative splicing. Increasing the frequency of these fibres may offer another therapeutic approach to reduce the severity of Duchenne muscular dystrophy. Antisense oligonucleotides have been shown to block aberrant splicing in the human β-globin gene. We wished to use a similar approach to re-direct normal processing of the dystrophin pre-mRNA and induce specific exon skipping. Antisense 2′-O-methyl-oligoribonucleotides, directed to the 3′ and 5′ splice sites of introns 22 and 23, respectively in the *mdx* pre-mRNA, were used to transfect myoblast cultures. The 5′ antisense oligonucleotide appeared to efficiently displace factors normally involved in the removal of intron 23 so that exon 23 was also removed during the splicing of the dystrophin pre-mRNA. Approximately 50% of the dystrophin gene mRNAs were missing this exon 6 h after transfection of primary *mdx* myotubes, with all transcripts showing skipping of exon 23 after 24 h. Deletion of exon 23 does not disrupt the reading frame and should allow the synthesis of a shorter but presumably functional Becker-like dystrophin. Molecular intervention at dystrophin pre-mRNA splicing has the potential to reduce the severity of a Duchenne mutation to the milder Becker phenotype.

Keywords: *mdx* mouse; Antisense oligonucleotides; Dystrophin pre-mRNA; Induced exon skipping; Duchenne muscular dystrophy; Genetic therapy

1. Introduction

Mutations in the human dystrophin gene are responsible for Duchenne muscular dystrophy (DMD) and the less severe, allelic form of Becker muscular dystrophy (BMD) [1]. The reading-frame hypothesis (as shown below) has supplied a molecular distinction between DMD and BMD phenotypes for the majority of cases [2]. Nonsense mutations, or genomic deletions/duplications which disrupt the reading frame of the dystrophin mRNA, lead to premature termination of dystrophin protein synthesis and the DMD pathology. In contrast, BMD mutations are generally found to be in-frame deletions where the reading frame is maintained, resulting in the synthesis of shorter but semi-functional dystrophin proteins.

The *mdx* mouse is an animal model of Duchenne muscular dystrophy [3]. It has been used to test therapies such as myoblast transfer and the introduction of a functional dystrophin gene, either directly in a plasmid or through a viral vector [4,5]. A nonsense mutation at base 3185 of the dystrophin gene transcript inactivates this gene in the *mdx* mouse, resulting in termination of translation within exon 23 [6]. No functional dystrophin should be expressed in *mdx*

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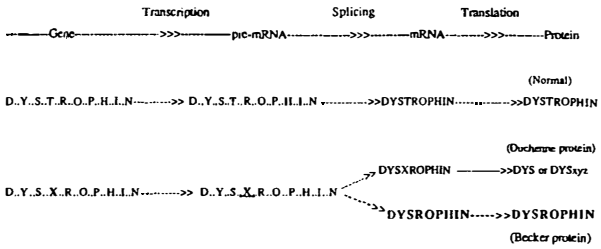
muscle but immunohistochemical staining has been used to detect rare, dystrophin positive-fibres (typically less than 1%) in *mdx* muscle tissue [7]. These fibres (called 'revertants' as they have reverted back to the normal dystrophin-positive state) have also been detected in many DMD patients [8,9]. It has been proposed that the dystrophin in these revertant fibres arises from an exon-skipping mechanism [10,11]. In the *mdx* example, alternatively-processed in-frame dystrophin gene transcripts skipping the exon carrying the primary nonsense mutation have been identified [12].

Antisense oligonucleotides (AOs) have been applied to down-regulate gene expression by blocking translation [13] or by targeting an RNA for RNaseH degradation [14]. AOs have also been used to target specific regions of DNA to inhibit transcription by RNA polymerase II [15,16]. In yet another approach, phosphorothioate 2′-O-methyl oligoribonucleotides, which do not induce RNaseH activity when bound to the RNA target, have been used to restore correct splicing in a thalassemic pre-mRNA by blocking aberrant splice sites [17,18].

The huge dystrophin gene spans 2.4 Mb and consists of 79 exons which must undergo extensive pre-mRNA processing to produce the mature mRNA of 14 000 bases [19]. Although induction of specific genomic deletions does not seem currently practicable, the potential exists for intervention during the processing of the dystrophin pre-mRNA. Two other groups have already demonstrated the very different applications of AOs to re-direct dystrophin gene transcript processing. Pramono et al. induced efficient skipping of dystrophin exon 19 in normal human lymphoblastoid cells by directing AOs to the exon recognition site of exon 19 [20]. Although expression was at very low levels in these cells, the dystrophin Kobe mRNA (skipping exon 19) was produced from a normal intact dystrophin gene. Rather than inducing a disease-associated dystrophin mRNA, Dunckley et al. used AOs directed at the splice sites flanking exon 23 of the *mdx* dystrophin gene to remove this exon (and the *mdx* nonsense mutation) from the mature mRNA [21]. The AOs used by this group did not appear to be efficient or specific as only very low levels of one alternatively-processed dystrophin transcript (with skipping of exons 22–29) were detected.

We describe an independent study of the application of AOs to induce efficient skipping of exon 23 during the processing of the *mdx* pre-mRNA in cultured myotubes. An *mdx* dystrophin gene transcript skipping exon 23 would be in-frame and could thus be translated into a slightly shorter dystrophin. As outlined below, an AO-based approach could also be used to induce skipping of one or more exons that flank genomic deletions in order to restore the reading frame of a DMD mRNA so that the phenotypic consequences of serious mutations in the dystrophin gene could be minimized. If such a therapeutic approach could be efficiently applied, a boy with a DMD genotype (e.g. 'DYS' nonsense or 'DYSxyz' frameshift mutations) may only develop a milder 'DYSROPHIN' Becker phenotype.



2. Materials and methods

2.1. Splice site characterization

DNA was prepared from normal and *mdx* mouse liver samples using the DNA Direct kit (Dynal). All oligonucleotides used in this study, either for PCR, sequencing or antisense studies, are shown in Table 1.

Long range PCR (Perkin Elmer rTth XL) was carried out across dystrophin introns 22 and 23 using primers based on the dystrophin cDNA sequence (primers Ex22F and 24R). PCR products were purified from unreacted primers and nucleotides using QIAquick PCR spin columns (Qiagen) according to the manufacturer's protocol. This material was partially sequenced with the original PCR oligonucleotides and primers Ex23F and Ex23R to establish the sequence of splice sites flanking exon 23. Direct sequen-

Table 1
Oligonucleotides used in this study

Application	Name	Sequence shown 5′ to 3′
XL-PCR/sequencing	Ex22F	AGTAGCATCAGGACGTG-GATCC
XL-PCR/sequencing	Ex22R	GAAAACATCAACTTCAGC-CATCC
PCR/sequencing	Ex23F	CTCTGCAAAGTTCTTTGAAA-GAGTAA
PCR/sequencing	Ex23R	GCTAGAAGAACATATGAA-TAAACTTC
PCR	Ex20Fo	CAGAATTCTGCCAATTGCTGAG
PCR	Ex26Ro	TTCTTCAGCTTGTGTCATCC
PCR/sequencing	Ex20Fi	CCCAGTCTACCACCCTATCA-GAGC
PCR/sequencing	Ex26Ri	CCTGCCTTTAAGGCTTCCTT
Antisense	AO dys 3′	CUUUGCAGAGCCUCAAAA
Antisense	AO dys 5′	CGGCUUACCUGAAAUUUUGG
Antisense	AO anti-globin	GCUAUUACCUUAACCCAG

Oligonucleotides for PCR and sequencing were synthesized on a Cyclone Plus DNA Synthesizer (Milligen) and desalted on NAP-10 columns (Pharmacia Biotech) after cleavage from the support. HPLC-purified antisense phosphorothioate 2′-O-methyloligoribonucleotides were obtained from Hybridon, Worcester, MA, USA. Note that this backbone avoids RNaseH activity directed at the RNA hybridized to AOs. The random AO represents a preparation synthesized as a library of all possible 18-mers.

cing of the PCR templates was carried out using the Prism dye-terminator chemistry and a 373A DNA sequencer (Applied Biosystems), as described in the manufacturer's protocol.

2.2. Cell culturing and transfection

C2C12 cells (ATCC # CRL-1772) were thawed from liquid nitrogen storage and grown in high glucose DMEM + 12% fetal bovine serum at 37°C 5% CO_2 in 24-well microtitre plates. The culture medium was replaced with 500 μM DMEM + 5% fetal bovine serum 24 h prior to transfection to encourage myotube formation.

Primary *mdx* myoblast culture preparation. Muscle was removed from four 6 week old *mdx* mice, washed three times in Hank's balanced salt solution (HBSS), minced with scissors in DMEM containing collagenase (0.15%) and incubated with mixing for 30 min at 37°C on a shaker. After the addition of an equal volume of phosphate buffered saline (PBS) to the slurry, cells were pelleted by centrifugation at 350 × *g* for 10 min. The soft cell pellet was resuspended in DMEM containing 12% foetal calf serum (FCS) and plated into gelatinized flasks for 4–6 days. To enrich the preparation for myoblasts, the cells were harvested by trypsinization and resuspended in 5 ml of calcium and magnesium free HBSS buffered with 20 mM HEPES and layered over 5 ml of 26% Percoll in HBSS which had been previously layered over 5 ml 34% Percoll in HBSS. This was centrifuged at 350 × *g* for 15 min at 20°C. Myogenic cells were removed from the 0:26% Percoll interface and the base of the 34% layer and diluted with an equal volume of HBSS before centrifugation at 350 × *g* for 10 min at 20°C. The cells were resuspended in high glucose DMEM supplemented with 5% fetal bovine serum and plated onto gelatine coated 24-well culture dishes.

2′-O-methyloligoribonucleotides, synthesized by Hybridon, were resuspended in sterile water to a final concentration of 1 mg/ml. AO/Lipofectin™ complexes were prepared as follows. Oligonucleotides and Lipofectin™ (Gibco BRL Products) were diluted separately to a total volume of 50 μl each in HEPES buffered saline (HBS; 20 mM HEPES, cell culture grade, 150 mM NaCl, pH 7.4). The two solutions were mixed gently in a polystyrene tube and left at room temperature for 10–15 min to allow formation of the AO/liposome complexes. This mix was then slowly added to the cultures with gentle agitation. Cultures were immediately returned to the incubator at 37°C, 5% CO_2 until RNA extraction was undertaken.

2.3. Reverse transcriptase PCR

Total RNA was prepared from cultured myotubes using RNAzol B (Tel-Test) as described by the manufacturer. cDNA was prepared in a 30-μl reaction where 1 μg of RNA was primed with 400 ng random hexamers and reverse transcribed with 100 U M-MLV reverse transcriptase (Promega). The reaction was heated to 94°C for 2 min and 70 μl of water was added. Five microlitres of the cDNA was then used as the initial template for a nested PCR with outer (Ex20Fo and Ex26Ro) and inner (Ex20Fi and Ex26Ri) sets of primers to amplify exons in the region of the *mdx* mutation using AmpliTaq Gold (Perkin Elmer). After an initial denaturation step of 94°C for 7 min, 40 cycles of 94°C for 30 s, 55°C for 1 min and 72°C for 2 min were carried out in an MJ minicycler. A 1-μl aliquot of the primary PCR was re-amplified in a 50-μl reaction for 30 cycles using the inner primer set.

3. Results

3.1. Characterization of dystrophin splice sites and design of AOs

Amplification of genomic DNA using primers Ex22F and 24R generated a single product of approximately 4.0 kb. Introns 22 and 23 were found to be 900 and 2850 bp long, respectively. Limited DNA sequencing of this material identified the splice sites flanking introns 22 and 23 (Fig. 1). GenBank accession numbers are AFO62828, AFO-62829, AFO62830 and AFO62831. Rodent splice site efficiencies were calculated [22]. Characterization of these splice sites allowed the design of 2′-O-methyl dys 3′ and dys 5′ AOs which would anneal to the 3′ and 5′ splice sites of introns 22 and 23 of the mouse dystrophin pre-mRNA respectively (Fig. 1). Control AOs were either a random sequence or targeted to the aberrant splice site in human β-globin pre-mRNA.

3.2. Induced exon 23 skipping during dystrophin pre-mRNA processing in C2C12 cells

The AO-approach was first tested on the immortalized mouse myoblast cell line C2C12, which can be induced to fuse and express normal dystrophin mRNA. Differentiating cells were transfected with a mixture of dys 3′ and dys 5′ AOs complexed with Lipofectin™ and incubated as indicated. Dystrophin mRNA was examined after reverse transcription-PCR across exons 20–26 to detect any skipping of not only exon 23 but also adjacent exons. A dose-dependant accumulation of the exon 23-deleted transcript was observed at 48 and 96 h after transfection (Fig. 2). Skipping of exon 23 was confirmed by direct sequencing of the appropriate PCR products (Fig. 3b–d). All C2C12 cultures treated with 50 nM or more AO mixture consistently generated dystrophin transcripts that skipped only exon 23. This transcript reached significant levels at 200 nM of both AOs, while at 400 nM additional shorter transcripts became evident suggesting unspecific effects of these oligonucleotides. Note however, that these shorter transcripts were more sporadic and not reproducible in all cultures. These shorter 'sporadic' transcripts were identified as exon 20 being

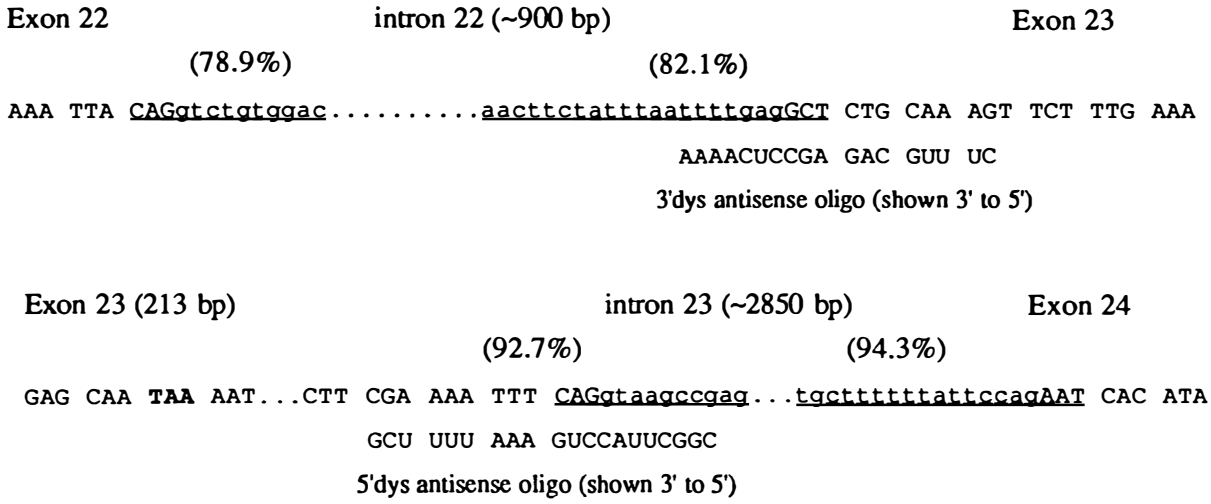


Fig. 1. Partial DNA sequence of the exons 22, 23 and 24 (capitals) and introns 22 and 23 (lower case). Splice sites have been underlined with calculated splice site efficiencies shown in parentheses. The reading frame is indicated and the *mdx* nonsense mutation is shown in bold. The antisense oligonucleotides to the 3′ and 5′ splice sites flanking exon 23 of the mouse dystrophin pre-mRNA are shown.

spliced to exon 24 and exons 21–24. It is not clear why the incidence of these transcripts increased in the presence of higher concentrations of both AOs. It is possible that disruption of the precise order of intron removal allowed other sequences, such as splice sites of exons 20 and 24, to compete more effectively for the splicing factors. More detailed experiments were carried out on primary muscle cells isolated from *mdx* mice.

3.3. Induced exon 23 skipping during dystrophin pre-mRNA processing in mdx cells

Primary *mdx* myoblast cultures were transfected with AO/Lipofectin complexes at a final concentration of 400 nM. Only the dys 5′ AO was found to induce skipping of exon 23 from the *mdx* dystrophin gene transcript (Fig. 4). This effect was observed consistently and the exonic arrangement of the shorter PCR product was confirmed by direct DNA sequencing as the splicing of exon 22 to exon 24 (Fig. 3b). Other AOs (dys 3′, globin, and random) had no apparent effect on dystrophin gene transcript processing. At a final concentration of 400 nM, these other AOs routinely generated only the normal dystrophin products as assayed by PCR. It has been observed that AOs directed at the 5′ splice sites tended to be more efficient at influencing splicing of pre-mRNA transcript [17,18].

Amplification across dystrophin exons 1–10, 40–50 and 60–70 did not detect any consistent alternative splicing in *mdx* myoblast cultures transfected with the dys 5′ AO (results not shown). No oligonucleotide-dependent cyto-

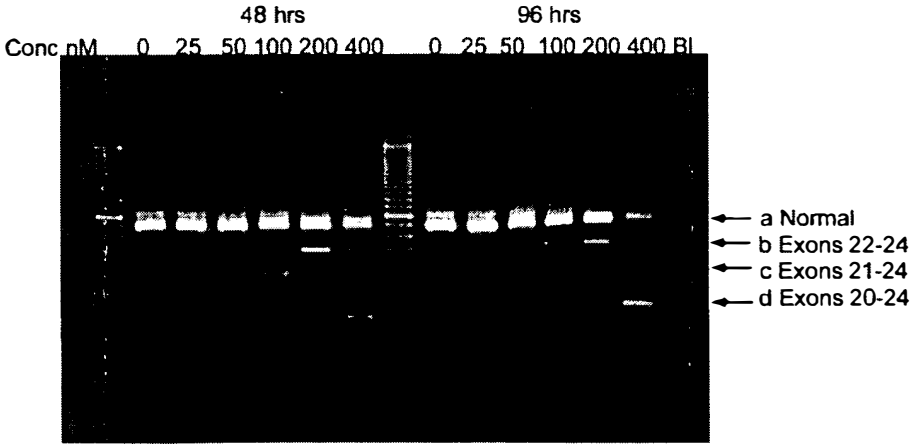


Fig. 2. Dose-dependent appearance of alternatively-processed dystrophin transcripts in C2C12 cells transfected with both dys 5′ and 3′ AOs and incubated for 48 or 96 h. AO/Lipofectin complex concentrations are indicated. The expected full length product is 901 bp and transcripts skipping exon 23 are 688 bp. Smaller fragments were identified as alternatively-processed transcripts where exons 20 or 21 were joined to exon 24.

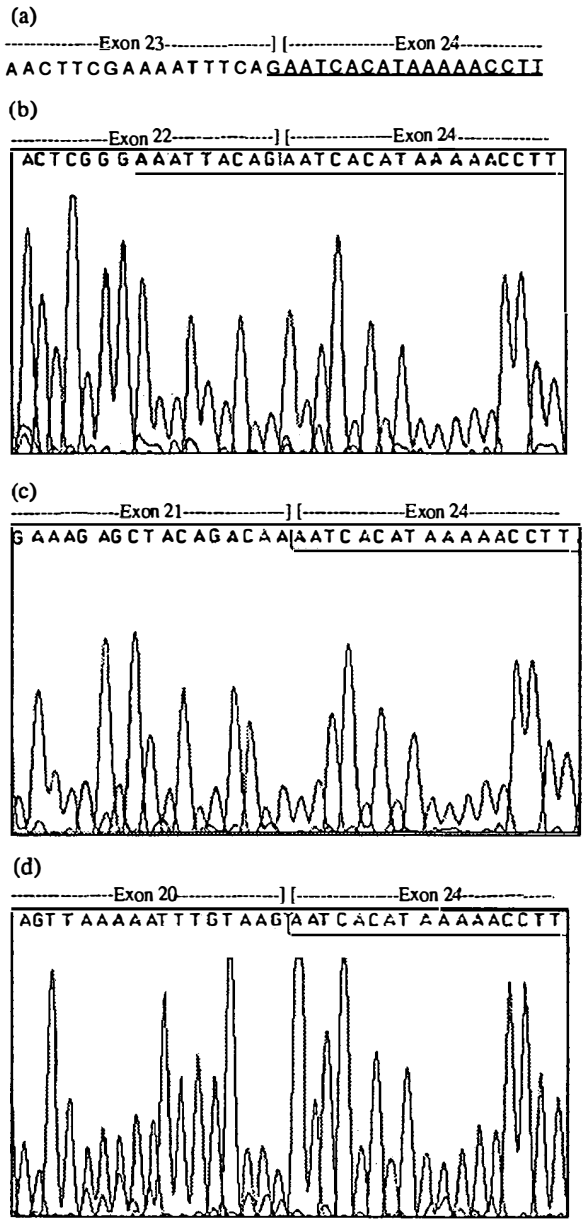


Fig. 3. Sequence of normal and induced alternatively-processed dystrophin gene transcripts. (a) DNA sequence across the junction of dystrophin exons 23 and 24. Chromatogram of the gene transcript across: (b) exons 22 and 24 (skipping exon 23); (c) exons 21 and 24 (skipping exons 22 and 23); (d) exons 20 and 24 (skipping exons 21, 22 and 23).

toxic effects have been observed. Although other murine genes have not been examined, lack of cytotoxicity provides a first line of evidence that the dys 5′ AO does not lead to functionally deleterious aberrant splicing of a large number of pre-mRNAs transcribed from other genes.

Induction of exon 23 skipping in *mdx* myotubes appears to occur soon after the transfection with the 5′ dys AO/liposome complex (Fig. 5). The observation that only the exon-23-skipped transcript was detected 24 h after transfection indicates that the *mdx* transcript must have a half-life considerably shorter than the estimate of the human dystrophin mRNA of about 15.6 ± 2.8 h [23]. If it can be assumed that total exon 23 skipping was induced soon after the addition of the AO, the 1:1 ratio of normal to exon-23-skipped transcripts after 6 h would imply that the *mdx* transcript half-life was about 6 h. This estimate is also consistent with approximately 10% normal dystrophin transcript being detected 18 h (>3 half lives) after transfection with the dys 5′ AO.

4. Discussion

DMD and BMD are allelic X-linked diseases which affect about 1 in 3500 live male births where the severe, early-onset DMD cases are more frequent than the milder BMD patients. DMD is typically associated with less than 3% of normal dystrophin expression levels while BMD is seen with dystrophin levels of 10% or more [24]. The presence of any dystrophin, albeit of reduced quality and/or quantity in BMD patients, highlights the significance and ability of a shortened but semi-functional dystrophin to reduce the

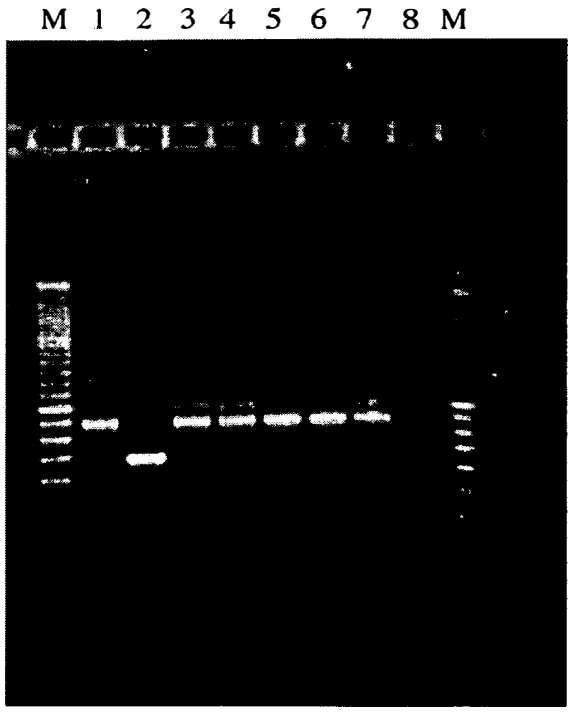


Fig. 4. Cultured *mdx* myoblasts/myotubes were transfected with AOs (400 nM)/Lipofectin complex (ratio of 1:5) and incubated for 24 h before RNA was extracted. RT-PCR was carried out across exons 20 to 26. M, 100 bp ladder; lane 1, dys 3′ AO; lane 2, dys 5′ AO; lane 3, anti-globin AO; lane 4, random AOs; lane 5, both dystrophin AOs (4 μm) delivered without Lipofectin; lane 6, HEPES buffered saline; lane 7, Lipofectin; lane 8, negative PCR control. Faint higher molecular weight products in some lanes represent carryover from the primary amplification reactions.

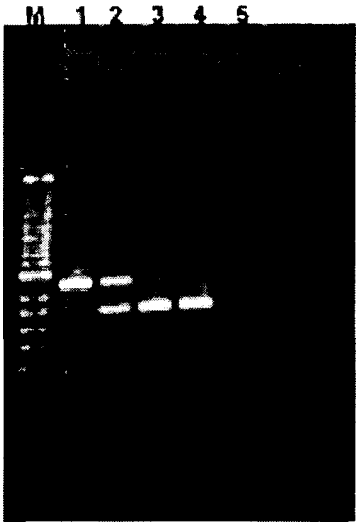


Fig. 5. Time course of exon-23-skipping after transfection of primary *mdx* cultures with the dys 5′ AO/Lipofectin complex. RT-PCR across exons 20–26 was carried out on RNA extracted from transfected cultures after 0 h (lane 1); 6 h (lane 2); 18 h (lane 3); 24 h (lane 4); negative PCR control (lane 5); M, 100 bp ladder.

severity of DMD. One study observed that DMD males with no dystrophin entered a wheelchair at a mean age of 7.9 years while those with detectable dystrophin were restricted to a wheelchair at 9.9 years [25].

There is a high de novo mutation rate in the dystrophin gene where one in three cases has no prior family history [26]. Although prenatal screening can reduce the frequency of DMD, new cases will continue to arise. Developing a therapy for this devastating disorder is imperative.

Gene or cell replacement therapies for DMD to date have relied on introducing the missing or defective gene product into tissues where the functional dystrophin is missing. The primary target tissue for cell/gene replacement has been skeletal muscle but it must be noted that dystrophin isoforms are also expressed in a variety of other tissues. The introduction of a muscle-specific isoform into muscle may address the problem of the missing protein in that particular tissue but may not replace missing isoforms expressed in other tissues.

Dystrophin expression is regulated by at least seven promoters in a cell specific manner so that multiple dystrophin isoforms are encoded by this one gene. Three distinct promoters at the 5′ end of the gene direct the synthesis of three high molecular weight isoforms (427 kDa) expressed in skeletal and cardiac muscle, Purkinje cells and neurons of the cerebral cortex [27–29]. Shorter isoforms derive from internal promoters and are expressed in a variety of tissues including the retina, central nervous system, peripheral nerve, liver, brain and testes [30–32]. Some of these isoforms would also be affected by a DMD/BMD mutation, depending upon the nature and position of the dystrophin gene defect.

Several studies have suggested that some DMD mutations have been by-passed via a naturally occurring exon-skipping mechanism. Immunostaining of individual revertant fibres from a DMD patient (genomic deletion of exons 35–43), using monoclonal antibodies against different parts of the dystrophin molecule, revealed five distinct immunoreactive dystrophin profiles [11]. These dystrophin-positive fibres appeared to have arisen from the removal of one or more exons flanking the primary deletion which then restored the reading frame of the dystrophin mRNA. Similar studies have also reported exon skipping in other DMD patients [10,33]. An examination of *mdx* mouse muscle tissue for alternatively-processed dystrophin gene mRNAs (that skipped exon 23 but maintained the reading frame) revealed at least four transcripts which could be translated into shorter but presumably functional dystrophin [12].

Dystrophin-positive or 'revertant' fibres have been observed in skeletal muscle of the *mdx* mouse and over 50% of DMD patients [7–10,33]. Since the dystrophin in these revertant fibres occurs naturally, there should be no immunological reactions to the revertant dystrophin if the frequency of these fibres was increased. The frequency of revertant fibres in humans is generally less than 1% although considerable variation has been reported [33]. Taken together, this would suggest a functional benefit of and selection for these dystrophin-positive fibres in dystrophic tissue. Similarly, as this revertant dystrophin appears to be correctly localized at the sarcolemma of the muscle fibre (based on immunostaining), the identification of the precise exon re-arrangements could provide a natural template for induced exon-skipping using AOs.

Initial AO experiments were undertaken in C2C12 cells, an immortalized normal mouse myoblast line, with the aim of inducing a minimal change to by-pass the *mdx* mutation, that is the skipping of exon 23. Targeted skipping of exon 23 was consistently induced in a dose-dependent fashion when both 3′ and 5′ AOs were delivered to those cells. Skipping of only exon 23 was never observed in any untreated C2C12 cultures. It should also be noted that during a search for alternatively-processed dystrophin gene transcripts from skeletal muscle of *mdx* and normal mice, dystrophin transcripts skipping only exon 23 were never observed [12]. Similarly, other transcripts detected in AO-transfected C2C12 cells (skipping of exons 21–23 and 22–23) had never been seen in normal or *mdx* muscle tissue, again indicating these transcripts had arisen from the action of AOs on the processing of the dystrophin pre-mRNA. Low levels of exon-23-skipping were detected in C2C12 cells after transfection with only the dys 5′ AO. The dys 3′ AO had no apparent effect on splicing in C2C12 cells. The inability of the dys 3′ AO to induce exon-skipping suggests that the splicing factors binding to the 3′ splice sites are more difficult to displace than their 5′ counterparts [17]. The fact that the dys 3′ AO was two nucleotides shorter than that directed to the 5′ splice site may have also contributed to this result.

Transfection of cultured *mdx* cells with both dys 3′ and 5′ AOs also resulted in the skipping of exon 23 but with a higher efficiency. Not only was exon-23-skipping complete 24 h after transfection but other alternatively-processed transcripts were not consistently generated. It was found that exon-23-skipping could be efficiently induced in transfected *mdx* myoblasts using only the dys 5′ AO. This was an encouraging result for potential clinical applications if minimal rearrangements of the dystrophin mRNA was to produce a functional protein. Attempts to detect or quantitate levels of dystrophin synthesis by Western blotting in transfected *mdx* cultures have been unsuccessful, presumably due to the relatively low numbers of myotubes in the primary *mdx* cultures.

Transfection of primary cultured *mdx* myoblast cells with a variety of dys 5′ AO concentrations also showed a dose-dependent result similar to that which had been observed in the C2C12 cells. However, in the *mdx* cells, the action of the dys 5′ AO appeared to be very specific in inducing skipping of exon 23. Other alternatively-processed transcripts detected in the transfected C2C12 cells were not detected even at the higher concentrations of 200 and 400 nM (results not shown).

A combination of mechanisms may be responsible for this striking improvement of exon-23-skipping in *mdx* cells compared to C2C12 cells: (1) oligonucleotide-Lipofectin complex uptake by primary *mdx* myoblasts may be more efficient than that by immortalized C2C12 cells. Transfection efficiencies are influenced by cell densities; (2) the shorter half life of the *mdx* transcript could enhance the extent of exon-skipping. It has been well documented that mRNA species containing nonsense mutations have shorter half lives than the normal counterparts [34]; (3) the accessibility of the 5′ splice site to the oligonucleotide may be increased by the stop codon mutation in exon 23 of dystrophin pre-mRNA.

The human dystrophin gene transcript has been shown to be spliced at the 5′ end before transcription was complete, suggesting co-transcriptional splicing [35]. However, that study was not able to determine the precise order of intron removal from the dystrophin pre-mRNA and processing of introns does not necessarily occur in a sequential fashion. The preferred order of intron removal from the endogenous adenine phosphoribosyltransferase pre-mRNA in Chinese hamster ovary cells is intron three followed by two then four or one [36]. It is possible that the removal of intron 23 (with exon 23) could precede, and hence influence removal of, introns 21 and 22.

It has been demonstrated that naked DNA (plasmid) can be taken up by skeletal muscle cells without any special delivery mechanism [37]. Preliminary transfection of cultured *mdx* cells with the dys 5′ AO without a liposome delivery system did induce low levels of exon-23-skipping. However, this exon-skipping was inconsistent and only sporadically observed in cultures which were incubated with 4 μm dys 5′ AO. In *mdx* myotube cultures where the AOs had been complexed with Lipofectin (Gibco BRL) or DOSPER (Boehringer Mannheim), comparable levels of exon-23-skipping were detected. AO/Lipofectin was found most efficient at a ratio of 1:5 to 1:10 while AO/DOSPER exhibited similar efficiencies at ratios of 1:10 to 1:15 (nucleic acid to liposome).

It has been reported that exon-19-skipping in the human dystrophin mRNA can be induced by targeting an AO to the recognition sequence of that exon in lymphoblastoid cells [20]. This was a surprising result which was difficult to interpret as the authors appear to have used oligodeoxynucleotides as antisense agents. Oligodeoxynucleotides are rapidly degraded in the serum-containing culture media and their duplexes with RNA are cleaved by RNaseH, a ubiquitous cellular enzyme. Thus, the expected effect of these oligodeoxynucleotides is site specific cleavage of the targeted RNA rather than skipping of the targeted exon [38]. In contrast, phosphorothioate 2′-O-methyl-oligoribonucleotides used in this report are stable in culture and do not promote RNaseH cleavage of target RNA. Furthermore, targeting of the splice sites may be more efficient since not all exons include characterized recognition sequences.

Nevertheless, a number of similarities between the induced skipping of human exon 19 and *mdx* exon 23 were apparent. In both cases, exon-skipping was detectable after 6 h and complete after 24 h. Targeted exon-skipping in both examples appeared to be specific in that rearrangements in other parts of the dystrophin mRNA were not routinely detected. Similar AO concentrations (200 nM) were needed to induce complete skipping, as were the ratios of DNA to liposome. The efficiency of the AOs in inducing exon-skipping was greatly reduced in the absence of a liposome delivery system. These similarities would imply that parameters for efficient exon-skipping in the mouse could be readily extrapolated to human cells and to other regions of the dystrophin gene.

Dunckley et al. describe the modification of splicing in the *mdx* dystrophin gene transcript using a 12-mer directed at the 3′ splice site of intron 22 [21]. This short AO was reported to induce skipping of not only exon 23 but exons 23–29. We increased the scope of the reverse-transcriptase-PCR assays but failed to detect this exon 22:30 transcript in any of our *mdx* cultures transfected with the dys 3′ and 5′ AOs described in this report (results not shown). Equally puzzling was the observation that their AO directed at the 5′ splice site of intron 23 had no apparent effect on splicing. Their annealing site at the exon 23/intron 23 junction was displaced by one nucleotide to the dys 5′ AO described here. This discrepancy could be due to either the highly specific nature of these AOs or could reflect transfection efficiencies. Dunckley et al. noted that while all myotubes demonstrated a relatively high transfection efficiency, based on the uptake of a FITC-labelled oligonucleotide, only 1–2% expressed dystrophin suggesting that the annealing site of the AO was a crucial element. Although we have not used a

fluorescent labelled AO to estimate transfection efficiencies, we have detected similar levels of induced exon 23 skipping with several commercial delivery systems (Lipofectin, Gibco BRL Products; DOSPER: Boehringer Mannheim; DOTAP, Boehringer Mannheim). We have not evaluated the transfection method described by Dunckley et al. [21].

All gene and genetic therapy approaches face the challenge of achieving efficient delivery. This issue will also need to be addressed in the delivery of AOs. However, advantages of an AO-based approach to the suppression of DMD dystrophin gene mutations include the slow turnover of the muscle fibres, the natural ability of these cells to take up DNA and the potential for repeated and systemic delivery. The apparent specificity of the dys 5′ AO, at least in *mdx* myotubes would suggest that systemic delivery may be possible with targeted dystrophin exon-skipping only being significant in those cells expressing dystrophin. As long as the AOs do not induce an immune response or become toxic, repeated systemic delivery should be possible. If the antisense sequences are incorporated into vectors that lead to stable expression of antisense RNA, the latter concern would be eliminated [39].

In summary, AOs may be regarded as a highly specific nucleic acid drug that re-programs existing cellular machinery to by-pass or minimize the consequences of serious dystrophin gene mutations. This antisense therapy has the potential to reduce the severity of the disease so that a boy with a DMD genotype would only develop a milder Becker phenotype.

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EXHIBIT 33

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Antisense oligonucleotides, exon skipping and the dystrophin gene transcript

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Antisense oligonucleotide induced exon skipping has recently emerged as a potential therapy to by-pass the consequences of many, but not all dystrophin mutations that lead to Duchenne muscular dystrophy. Targeted removal of one or more exons, to restore a disrupted reading frame, or omit a nonsense mutation, could lessen the consequences of an estimated 80% of dystrophin gene mutations. Promising *in vitro* and *in vivo* experiments in animal models of dystrophinopathies, as well as demonstration of induced exon skipping in cultured human myogenic cells have prompted considerable enthusiasm. Furthermore, advances in antisense oligonucleotide chemistries have resulted in the development of more stable and less toxic compounds, some of which are currently in Phase III clinical trials for selected antiviral applications. This review will summarize developments in induced exon skipping that have paved the way to clinical trials and some of the challenges and possible limitations.

Keywords: Alternative splicing, Revertant Fibres, Mutation suppression, Duchenne muscular dystrophy

Introduction

Mutations in the dystrophin gene that preclude the synthesis of a functional protein lead to Duchenne muscular dystrophy. In developing a treatment for DMD, compensating for the defective dystrophin gene has now been recognised as a much greater challenge than originally anticipated. Potential therapies have included cell (myoblast, satellite and stem cell) or gene replacement (viral and non-viral delivery) (for review see [1,2], read-through of nonsense mutations [3,4], corticosteroids [5-8] or inhibition of specific proteolysis [9,10] and the subject of this review, antisense oligonucleotide (AO) induced exon skipping. Of all these approaches, it is only the latter that has any natural precedent.

Dystrophin positive revertant fibres [11] in dystrophic tissue arise from an unknown exon skipping mechanism [12-14], while the variable phenotypes observed in Becker muscular dystrophy patients clearly demonstrate that some in-frame, internal deletions of dystrophin, particularly in the rod domain, can result in a protein of near normal function [15-17].

Furthermore, although chemically synthesised antisense oligonucleotide (AO) analogues cannot be regarded as natural compounds, small, naturally occurring, non-coding RNAs have been identified and implicated in the control of a variety of cellular processes [18]. Small RNAs have been shown to silence selected genes [19] and modify gene expression at the level of splicing or translation [20]. Therefore, the application of AOs to modify gene transcripts for therapeutic outcomes should not be regarded as whimsical.

Natural precedents for an Exon Skipping approach to address dystrophin mutations

Revertant fibres were reported in the *mdx* mouse [11] and in DMD patients [21] and so named because of 'reversion' to the normal dystrophin staining pattern. Various dystrophin mRNA transcripts excluding the primary genetic lesion, and in which the reading frame has been restored or maintained have been described in human, canine and murine dystrophic tissue [12, 22, 23]. It is now clear that revertant fibres result from an exon skipping mechanism, and that not all have the same exonic combination [13]. *In situ* hybridization studies using a dystrophin intron 21-exon 25 genomic probe on *mdx* mouse muscle showed that the dystrophin gene was intact in the majority of revertant fibres, and RT-PCR and antibody epitope mapping indicated that the most common exon skipping rearrangements involved 20 or more exons [13].

With the apparent exclusion of secondary somatic genomic deletions within the dystrophin gene being the cause of revertant fibres, the mechanism

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responsible for generating dystrophin is most likely to involve a localized alteration in splicing. Since revertant fibres occur singly or in small clusters, suggesting a clonal origin [11,13, 24], the events that bring about exon skipping must only occur within the dystrophin–positive fibres and not in the surrounding muscle. Small non-coding RNAs have recently been credited with controlling aspects of gene expression, from splicing to translation [19,20]. The possibility exists that the revertant fibres express novel microRNA variants that interfere with dystrophin pre-mRNA processing.

Apart from confirming the existence and utility of exon skipping in the dystrophin gene transcript, another important property of revertant fibres is that they not only demonstrate immune tolerance to dystrophin, but may also play a causative role in the development of this tolerance [25,26]. Depending upon the nature and position of the mutation, production of amino terminal fragments and dystrophin isoforms from internal promoters would also expose the immune system to various dystrophin epitopes. Consequently, an immune response to any induced dystrophin in individuals who have revertant fibres is considered unlikely, although the possibility of novel epitopes encoded by the induced exon junctions cannot be excluded.

The dystrophin gene rearrangements in mildly affected BMD patients clearly demonstrate that some domains are not essential for near-normal function. The reading frame rule [27] holds true for the majority of dystrophin mutations. Nonsense or frame shifting mutations result in premature termination of translation and the absence of a functional protein leads to DMD while in-frame deletions cause BMD [27]. In some cases, the consequences of a deletion are so mild that the individual is asymptomatic and may only be diagnosed later in life [16,17,28,29]. There appears to be an upper limit to the size of in-frame deletions that may be tolerated, where the loss of 34 or more exons is invariably associated with a severe phenotype [30].

Exceptions to the reading frame hypothesis and the need for precise mutation detection

Apart from rare mis-sense mutations in crucial binding domains of the dystrophin gene, many of the apparent exceptions to the reading frame rule may be explained when the responsible secondary mechanisms are identified. Some dystrophin nonsense mutations do not lead to DMD, since the base change compromises motifs involved in pre-mRNA processing [31,32]. In these cases, the nonsense mutation may prevent efficient exon recognition by the splicing machinery and the exon is variably excluded from the mature dystrophin mRNA. If loss of the exon does not disrupt the reading frame, the nonsense mutation is removed from the mature dystrophin gene transcript and a slightly shorter, BMD-like protein can be produced. The amount of functional dystrophin generated, and hence the severity of the phenotype, reflects the degree to which the exon is excluded [32]. If the effect of the base change were to marginally weaken splicing, generating only a small percentage of the transcripts missing the mutation, a more severe phenotype would be predicted. Conversely, if exon skipping as a result of the nonsense mutation was complete and assuming that the lost coding domain was not essential, the patient could be asymptomatic.

It has been estimated that some 15% of human mutations alter splicing [33]. Changes in primary splicing motifs that may be readily identified include, the branch-point, acceptor and donor splice sites. Other DNA changes that may alter splicing can be less obvious, particularly when a single base change deep within an intron results in the inclusion of a pseudo exon [34]. Intronic changes over 10 kilobases from the nearest coding sequence have been shown to alter the processing of dystrophin exons [35]. Exonic splicing enhancers, motifs recognised by splicing factors such as the SR-proteins can be predicted *in silico* [36] but accurate identification occurs when a particular exonic base change modifies the splicing pattern [37]. An apparently neutral polymorphism (C>T change at the third base of codon 608 in the lamin A/C gene) is responsible for Hutchinson-Gilford Progeria Syndrome [38-40]. This *de novo* substitution activates a cryptic splice site 5 bases upstream that leads to the loss of 150 nucleotides from the gene transcript [40].

It is examples such as these that emphasize the need for detailed molecular characterization in disease diagnosis, so that not only are DNA changes detected, but the consequences of the alterations are considered. Furthermore, precise mutation detection will be essential prior to the application of targeted therapies such as splicing manipulation. The boundaries of the genomic deletions or duplications must be clearly defined so that the appropriate target site can be characterized for the design of AOs to restore the reading frame. Similarly, any exon carrying a

nonsense mutation must be identified and a strategy developed to remove that exon, and flanking exons if necessary to maintain the reading frame. Many exons in the dystrophin gene, particularly those encoding the rod domain, would not disrupt the reading frame if excluded. Excision of a single exon should be sufficient to overcome a majority of the prematurely truncating mutations. It may also be necessary to confirm the sequence of the AO annealing site to eliminate the possibility of natural DNA variation compromising AO annealing.

Mechanics of Antisense Oligonucleotide manipulation of gene expression

AOs were originally applied to down-regulate specific gene expression, most commonly through the induction of RNaseH, an enzyme that degrades the RNA strand of an RNA:oligodeoxyribonucleotide duplex. Unmodified oligonucleotides have a natural phosphodiester backbone that renders these compounds susceptible to nuclease degradation. One chemical modification to increase oligonucleotide stability is the substitution of the non-bridging oxygen atom of the backbone with sulphur [41]. The resultant phosphorothioate (PS) backbone confers greater resistance to degradation, but concomitantly elicits some non-antisense effects [42].

Additional increases in stability were achieved by modifying the ribose moiety of the oligonucleotide. Advantages of the 2'-O-methyl (2OMe) modified bases on a PS backbone include greater resistance to nuclease degradation, but perhaps even more significantly, these compounds do not induce RNaseH activity when annealed to an RNA target [43,44]. Instead of targeting an mRNA transcript for RNaseH degradation, appropriately targeted 2OMeAOs bind to motifs in the primary gene transcript and may interfere with exon recognition and/or spliceosome assembly [45].

It is possible, therefore to alter gene expression through a variety of mechanisms. In addition to RNaseH degradation of a target gene transcript, gene down-regulation may be brought about by AOs directed to motifs involved in translation. Suppression oftranslation by AOs with novel backbone chemistry, phosphoro-amide morpholino oligonucleotides (PMOs) [46,47], has been very effective in a number of systems [48-50]. PMOs directed upstream of the initiation codon of the target mRNA are able to prevent ribosome binding and subsequent protein translation [51]. These compounds have an uncharged backbone and exhibit remarkable biological stability, as there are no enzymes that can recognize and degrade the morpholine structure [46]. Metabolic breakdown products of PMOs have not yet been detected and the uncharged backbone does not appear to exhibit non-antisense effects, as do AOs on PS backbones. Several PMOs are under evaluation as antiviral agents with promising results, and a PMO has entered clinical trials [52], http://www.antivirals.com/devNeugene.html and no overt adverse effects reported to date.

In application to both splicing and translation suppression, AOs must be carefully designed to the susceptible target motifs and should preferably be resistant to degradation. An AO with a brief biological half-life may only affect splicing or translation for a limited period and changes in expression may not be readily detectable. Biologically stable AOs would exert a more sustained effect, depending upon the half-life of the gene product and the turnover of the tissue. Improvements in the design and synthesis of AOs, have yielded novel chemistries, including PMO which show no overt adverse effects [53-55] and persist and exert biological activity for extended periods [56]. The major isoforms transcribed from the dystrophin gene are relatively stable and expressed in cells with slow turnover, and it is not unrealistic to expect that periodic administration of AOs could induce and maintain therapeutic benefits.

Another factor to be considered is the extent to which gene expression must be altered to obtain the desired benefit. In targeting a viral or oncogene transcript for suppression, the degree of gene down-regulation may need to be absolute to achieve therapeutic benefit. Conversely, in order to alter the course of a disease such as DMD, only a proportion of gene transcripts may need to be modified. Defective dystrophin gene transcripts cannot encode a functional protein and are likely to be subjected to nonsense mediated decay, while any modified in-frame transcripts would undergo repeated rounds of translation and allow the protein to gradually accumulate with time.

AO modification of splicing

Richard Kole (Chapel Hill, North Carolina) pioneered the application of AOs to address splicing mutation in the β-globin gene [57-59]. These researchers used AOs to mask cryptic splice sites in the introns of the β-globin gene transcripts to normal-

ize the splicing patterns. This concept has been re-interpreted to address DMD. Normal splice sites of selected exons are targeted to induce abnormal dystrophin pre-mRNA processing to bypass mutations, which would otherwise prematurely terminate translation.

The first report of AO-induced exon skipping in the dystrophin gene transcript was from the laboratory of Professor Masafumi Matsuo, (Kobe Japan), who used an oligodeoxyribonucleotide (ODN) of 31 bases directed at a motif in human dystrophin exon 19 [60]. Exon 19 skipping was induced in human cultured lymphoblastoid cells, although oligonucleotides of this chemistry are more commonly used to induce degradation of target mRNA. A likely explanation for this is that once the RNA/ODN duplex was excised from the transcript, along with the flanking sequence, the resultant mRNA would no longer be susceptible to RNaseH degradation.

A variety of AO chemistries have now been evaluated to modify dystrophin exon processing and the most commonly used to date are the 2OMeAOs [61-67]. Recently, we reported that 2OMeAOs were much more effective at inducing dystrophin exon skipping than ODNs of identical sequence in *in vitro* studies [68]. It is now apparent that PMOs are even more efficient than the equivalent 2OMeAOs when administered by intramuscular injections in the *mdx* mouse model of muscular dystrophy [56].

Design of AOs for induced exon skipping

Obvious targets for AO intervention are the acceptor and donor splice sites. Intraexonic sites involved in exon recognition and splicing are called exonic splicing enhancers (ESEs) and these can be predicted to some extent *in silico* [36]. Errington et al [69] undertook a study of splicing motifs within, and flanking exon 19 that could be targeted to induce skipping of that exon. Exon 19 was found to be readily omitted from the mature human dystrophin gene transcript after application of AOs directed at the acceptor and donor splice sites, as well as shorter AOs targeting motifs within the 31 nucleotide domain identified by Pramono et al, [60] as containing an ESE. An AO of only 14 nucleotides was able to induce exclusion of exon 19, albeit only after administration at relatively high concentration [69].

Once a suitable motif that can be masked to disrupt spliceosome assembly and induce consistent and sustained target exon skipping has been identified, additional options become available to further enhance the biological effect. These include terminal modifications [68], other chemistries [70-73] and agents to enhance uptake [65,74].

Judith van Deutekom, and collegues [2,73,75-78] have undertaken extensive studies on suppressing many DMD mutations. In addition they have also developed a "humanized transgenic mouse" which carries the entire human dystrophin gene [79]. Although the human dystrophin exons are recognised and correctly processed by the murine splicing machinery, an important outcome of this work was the demonstration of the exquisite sensitivity of AOs in targeting specific sites. AOs directed at the human gene transcript could modify splicing of that transcript, but still have no effect on the corresponding region of the murine dystrophin mRNA. Although AOs may be regarded as exerting comparable specificity to that of a PCR primer, the potential of an AO to cross-react with a homologous but unrelated mRNA cannot be discounted. However, this may not prove to be of any great concern. It has been our experience in designing AOs for dystrophin exon skipping that moving an oligonucleotide target site by only a few nucleotides can alter the biological effect by an order of magnitude (unpublished observations).

Clinical trials

An ENMC workshop was held in Naarden, the Netherlands to discuss clinical trials using AOs in DMD [80]. At the time, the most advanced plans were presented by the Dutch/Belgian group, who propose to exclude exon 51 as the initial target. This was to be undertaken with a 2OMe AO on a phosphorothioate backbone directed at an exon 51 ESE. The therapeutic compound will be injected, in the absence of delivery enhancing agent, as a single dose in several injections in the *tibialis anterior*. A biopsy will be taken 28 days later for analysis of RNA and protein. Patient recruitment is currently ongoing and the trials are expected to be completed by during 2006.Trials in the United Kingdom would be of a similar nature, but with some variation to avoid direct duplication. Regardless of the fine details of the AO to be employed or the muscle to be treated, it was decided that there should be consistency and cross-validation of the results obtained in the studies.

Nucleic acids, including AOs, are generally not efficiently taken up by cells, particularly in *in vitro* studies. A number of agents are available that can facilitate intra cellular delivery of AOs,

including polyethylenimine, cationic liposomes and block copolymers. It is important to note that not all of these agents perform similarly *in vitro* and *in vivo*. Lipofectin or Lipofectamine 2000 can deliver charged AOs into cultured cells or muscle with, but at a cost of moderate cell damage. The block copolymer F127 has been used to introduce 2OMeAOs *in vivo* [65,66], however, this agent is ineffective *in vitro*.

The different AO chemistries will have specific requirements for cell delivery and nuclear uptake. The delivery, and subsequent exon skipping efficiency, of 2OMeAOs is substantially improved by the use of agents to enhance delivery and uptake [63-65]. We recently reported that PMOs can be administered *in vivo* without a delivery agent, a feature that should facilitate their use in clinical trials. Data using a PMO to by-pass the dystrophin exon 23 mutation in the *mdx* mouse have indicated this chemistry to be superior to the 2OMeAOs [56]. Under parallel conditions, uncomplexed AOs were administered to mice by intramuscular injection and only the PMOs were able to induce persistent dystrophin expression. In contrast, the equivalent 2OMeAO was unable to generate any correctly localized dystrophin as determined by immunohistochemical staining or western blotting. The 2OMeAO, when administered at higher concentrations did induce some exon skipping, however, at these dosages, the AOs are more likely to cause adverse or non-specific effects.

The Future

The use of AOs to alter gene expression by redirecting splicing should not be regarded as a 'gene therapy'. Although synthetic nucleic acid analogues can manipulate the processing of the pre-mRNA, the DNA remains unaltered and no permanent genetic changes are introduced. Once the AO has been removed from the system, the exon skipping effect will be lost and any benefit derived from the treatment will only persist while the modified gene transcripts and the induced, internally shortened dystrophin protein remains in the cell. It has become clear from several studies that dystrophin is a particularly stable protein and, since muscle fibres have a slow turnover, AO induced dystrophin may be correctly localized for weeks or months after a single treatment [56,66,67].

Nevertheless, if exon skipping does prove to be a viable therapy, periodic administration of AOs will be essential to maintain clinically beneficial levels of dystrophin. The frequency of AO re-administration required will depend upon a number of variables, including the route of administration, efficiency of uptake as well as persistence and duration of AO action. Based upon current animal studies using PMO preparations, we postulate that a weekly injection regimen of between 5 and 25 mg PMO per kg will be required to induce detectable dystrophin that would have the potential to ameliorate the severity of DMD. The prolonged half-life of this protein and the slow turnover of muscle fibres would suggest that a maintenance dose would then be necessary, possibly at less frequent intervals. Potential delivery regimens include intravenous and subcutaneous delivery, however investigation on modes of further enhancing and improving systemic delivery is ongoing.

Repeated administration of AOs to maintain dystrophin expression is not without risk. No information on the consequences of long-term administration of PMOs or other antisense chemistries to humans is currently available. Although PMOs appear to be the chemistry of choice for the initial AO DMD trials, it is imperative that other chemistries continue to be evaluated, in the event that there are unanticipated adverse effects associated with these compounds.

Exon skipping can be induced by natural RNA, using adeno-associated viral constructs containing expression cassettes coding for antisense RNA sequences under the control of U7 promoters. Goyanville et al., [81] clearly demonstrated the utility of this approach in the *mdx* mouse. This work has recently been successfully extended to the canine golden retriever muscular dystrophy model. Although extremely promising, viral AO delivery must be regarded as a gene therapy and confront the challenges associated with the use of viral vectors, such as achieving sustained expression, overcoming immunological reaction and high production cost.

Concluding comments

Luis Garcia (Genethon, France) has referred to the exon skipping strategy as an "a la carte" therapy. Unique compounds will be required to address the numerous mutations occurring across the dystrophin gene, but this may not prove to be an onerous task because of the existence of hot-spots for genomic deletions. It has been proposed that a small number of AOs would address the majority of these dystrophin deletions [76]. Nonsense mutations occur at an estimated frequency of 15% of DMD cases and

appear to be distributed evenly across the dystrophin gene. This will necessitate a panel of AOs that will allow removal of any dystrophin exon that does not code for a crucial functional domain, since the rod domain consists of almost half the coding sequence. The Perth laboratory has developed AOs to target over 50 exons in the dystrophin gene transcript. Although some exons remain to be addressed, and certain AOs require further optimisation, a number of potentially therapeutic sequences are now available for application in clinical trials.

Despite the enthusiasm and anticipation for the upcoming AO clinical trials, the expectations must be realistic. Exon skipping cannot "cure" DMD. At best, exon skipping may reduce the severity, with the extent of any benefit greatly influenced by many factors, including the age of the patient, the nature and position of the mutation, as well as the efficiency and persistence of the AO effect. However, if AO therapy does induce synthesis of some functional dystrophin in patients, a substantial delay in the progression of this disorder would be anticipated. The outcome of the AO therapy may be further improved if other treatments shown to be of benefit, including coticosteroids, are also included.

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